The NRSF and USF transcription factor families regulate pro-convulsant neuropeptides and are targets for anti-convulsant drug treatment: Implications for epilepsy.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Stuart Graham Gillies.

October 2009

The NRSF and USF transcription factor families regulate pro-convulsant neuropeptides and are targets for anti-convulsant drug treatment: Implications for epilepsy.

Stuart G Gillies

Epilepsy is a chronic neurological disorder which can arise following an initial insult that over time, progresses into a condition characterised by recurrent spontaneous seizures. During a latent period between the initial insult and the epilepsy condition proper, major changes occur within the brain at both a cellular and molecular level, in a process known as epileptogenesis. It is postulated that during epileptogenesis, signal transduction pathways are perturbed following the initial insult, which may bring about long term changes in gene expression profiles. For example, the expression of a host of neuropeptides is known to be modulated in response to an initial insult, including the up-regulation of the pro-convulsant tachykinins Substance P and Neurokinin B, encoded by the TAC1 and TAC3 genes, respectively. In this thesis I have explored the regulation of both of these genes by two distinct transcription factor (TF) families; the Neuron Restrictive Silencing Factor (NRSF) isoforms, and the Upstream Stimulatory Factor (USF) proteins. I demonstrate that both NRSF and USF variants regulate TAC3 promoter activity, and that NRSF isoforms can modulate endogenous NKB expression in a human neuroblastoma cell line. Furthermore, these distinct TF families are shown to work in cooperation to regulate the activity of the rat TAC1 promoter. Thus, both NRSF and USF variants are shown to be important in the regulation of pro-convulsant neuropeptides and as both NRSF and USF proteins have been shown to be induced by pro-convulsant stresses here, they are potential key TFs in epileptogenesis, responding to an initial insult, and orchestrating downstream gene expression changes.

Consistent with such a model, I have also revealed that both NRSF and USF variants are modulated by anti-convulsant drug treatment. Here, three distinct anti-convulsant drugs, were found to differentially modulate the expression of both the full-length NRSF, and its truncated isoform, as well as the USF proteins USF1 and USF2. Furthermore, whilst the drugs had limited impact upon the localisation of these TFs in human neuroblastoma cells, they did affect the binding of these TFs to target DNA sequences, particularly NRSF binding to its recognition DNA sequence, the NRSE, in a number of genes. In addition, due to an increasingly appreciation of the role of cocaine and the dopaminergic pathways in seizure progression, I explored the impact of cocaine treatment on the expression of these TFs. Cocaine was found to modulate both NRSF and USF variant expression, and NRSF binding to target DNA sequences. These findings suggest that both NRSF and USF variants are important in epileptogenesis and are targets for modulation by the anti-convulsant drugs investigated here. To further explore the significance of NRSF expression in seizure progression, I explored the impact of over-expression of NRSF isoforms, modelling that which occurs in response to seizure in animal models, on global gene expression pathways. I reveal that NRSF isoform over-expression significantly modulates the expression of a host of genes with known associations with epilepsy, supporting a model that NRSF isoforms are key TFs which respond to the initial insult and coordinate long-term changes in gene expression. These findings may help our understanding of the molecular mechanisms at work during epileptogenesis, and may better our understanding of the progression of epilepsy.

Acknowledgements

I would like to take this opportunity to thank the many people who have helped and supported me throughout my PhD. Firstly I would like to thank my supervisors Professor John Quinn and Dr Jill Bubb, for all their support, direction and friendship over the past three years, that has not only shaped my project, but also me as a person. I would also like to thank my lab colleagues in white block, most notably Dr Kate Haddley, Dr Sylvia Vasiliou and Dr Fahad Ali, who I now consider to be life-long friends. Their friendship and support helped me overcome all the barriers that a three-year PhD project presented, and for that I am extremely grateful. I would also like to thank Dr Helen Burrell, Mr Iain Kirk, Miss Hannah Stevens, Dr Moutasem Abo-onq, Dr Gregory Jacobson and Mr Steven Thompson, who have all helped me over the past three years. Finally, I would like to thank my PhD course organisers and past MRes supervisors who all helped shape me as a scientist, including Professor Andrea Varro, Dr David Criddle and Dr Sylvia Urbe. I also wish to thank the MRC for funding my PhD Studentship.

On a more personal note, I am fortunate to have a fantastic family and great number of friends who have all provided support for me over the past few years, whether it was listening to my moans, or just simply helping me to relieve stress with a few pints. I would like to take this opportunity to thank my parents for 27 years love, support and encouragement. They have worked hard throughout their lives to provide a platform for me to achieve my aspirations, and for that I am eternally thankful. I would also like to thank Bob, Nicola, Ste and Joe, who have always been there for me and provided much needed love and laughter. Finally I would like to thank my fiancée Catherine, who has lived through all the good times and the bad times with me, and has supported me through both. Catherine has been both my rock and inspiration throughout my PhD and I will always love her and be thankful for all she has done for me.

CONTENTS PAGE

Abbreviations

- ACD – Anti-convulsant drugs
- AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- AP1/CRE - activation protein 1/cAMP response element
- ATCC - American Type Culture Collection
- AVP - arginine vasopressin
- BD – Bipolar Disorder
- BDNF – Brain Derived Neurotrophic Factor
- bHLH - basic-Helix-Loop-Helix
- CART - cocaine and amphetamine-regulated transcript
- CBZ – carbamazepine
- CDYL - chromodomain on Y-like
- CGRP - calcitonin gene related peptide
- ChIP – Chromatin Immunoprecipitation
- CNS - central nervous system
- CREB - cAMP response element binding protein
- DMEMs - Dulbecco's modified eagle's media
- DMSO - Dimethyl sulfoxide
- DRG – Dorsal root ganglion
- ESCs- embryonic stem cells
- FBS - Foetal bovine serum
- GABA - γ-aminobutyric acid
- GPCRs - G protein-coupled receptors
- HAP-1 - Huntingtin-associated protein 1
- HAT - histone acetyltransferase
- HCN2 - Hyperpolarization activated cyclic nucleotide-gated potassium channel 2
- HD – Huntington's Disease
- HDAC - histone deacetylase
- HK-1 - hemokinin-1

- HMTs - histone methyltransferases
- HP1 - Heterochromatin protein-1
- hTERT - human telomerase reverse transcriptase
- IP – immunoprecipitations
- KA – Kainic Acid
- KCC2 – K-Cl cotransporter
- LMT – lamotrigine
- MeCP2 - methyl-CpG-binding protein 2
- MSCs - mesenchymal stem cells
- NEAA - non essential amino acids
- NGF – Nerve growth factor
- NK1R – NK1 receptor
- NK2R – NK2 receptor
- NK3R – NK3 receptor
- NKA – Neurokinin A
- NKB – Neurokinin B
- NMDA - N-methyl-D-aspartic acid
- NPK – neuropeptide K
- NPY – neuropeptide Y
- NPγ - neuropeptide γ
- NRSE – neuron restrictive silencing element
- NRSF - neuron restrictive silencing transcription factor
- PCR – polymerase chain reaction
- PHY – phenytoin
- PIC – Pre-Initiation Complex
- PICt – Protease Inhibitor cocktail
- PME - progressive myoclonus epilepsy
- PNS - peripheral nervous system
- Pol II - RNA Polymerase II
- qPCR – quantitative PCR

- RE-1 - Repressor element-1
- REST - Repressor element-1 silencing transcription factor
- RILP - REST/NRSF interacting Lin-11, Isl-1, Mec-3 (LIM) domain protein
- RT – Room Temperature
- RT-PCR - Reverse Transcriptase PCR
- SCLC – small cell lung cancer
- SCN2a - voltage-gated sodium channel, type II alpha
- SP – Substance P
- SSADH - Succinic semialdehyde dehydrogenase
- SVZ – subventricular zone
- TAF – TBP associated factor
- TBP – TATA binding protein
- TF – transcription factor
- TSS - transcription start site
- USF - upstream stimulatory factor
- USF1 - upstream stimulatory factor 1
- USF2 - upstream stimulatory factor 2
- VPA – Valproic Acid
- DG – Dentate Gyrus
- GTC - Generalised Tonic-Clonic
- PPS – Perforant path stimulation
- SE – status epilepticus
- SSSE – self-sustaining status epilepticus
- TLE – temporal Lobe Epilepsy

CHAPTER 1: General Introduction

1.1 Epilepsy

1.1.1 Social context

Epilepsy is one of the most commonly acquired, chronic, neurological disorders suffered worldwide. There are approximately 50 million people suffering with epilepsy, with nearly 90% of cases in the developing countries (WHO, January 2009). The incidence of epilepsy is quoted as being around 40-70 per 100,000 people, in developed countries, rising to 120 per 100,000 in undeveloped countries, whilst the prevalence rate is between 0.4-1% (Bell & Sander., 2001; De Boer *et al*., 2008). Unfortunately, despite the availability of a diverse array of anti-convulsant drugs (ACDs), approximately half of patients treated with modern ACDs continue to experience seizures (Pitkanen, 2002). Epileptic seizures are defined as clinical manifestations of abnormal, excessive or synchronous neuronal activity in the brain (Fisher *et al*., 2005). It should be noted that the presence of a single seizure, is not synonymous with having epilepsy, and that epilepsy is defined by the occurrence of at least one epileptic seizure, together with an enduring alteration of the brain (Fisher *et al*., 2005). Seizures can be one of two types, partial (focal) or generalised. Partial, refers to abnormal discharge in a localised group of neurons, resulting in altered senses, facial movements and twitches. Generalised seizures in contrast occur when generalised neuronal activity begins simultaneously in both brain hemispheres, and can result in the loss of consciousness, falls and involuntary muscular contractions known as ‘convulsions’.

Epilepsy is not a single condition, but a diverse family of disorders that share an increased "propensity to have seizures". This definition encompasses multiple forms of epilepsy including absence epilepsy and temporal lobe epilepsy (TLE). Absence epilepsies are defined as non-convulsive epileptic seizures where loss of consciousness occurs (Crunelli & Leresche., 2002), whilst TLE is characterised by recurrent unprovoked seizures, originating from the medial or lateral temporal lobe, consisting of a combination of simple or complex partial and secondarily generalised seizures, which may result in memory loss (Abou-Khalil *et al*., 1993; Briellmann *et al*., 2002). Some epilepsy disorders may be idiopathic, with no known cause (besides a possible genetic cause), such as idiopathic generalised epilepsy which can include generalised tonic-clonic (GTC) or 'grand-mal' seizures (seizure involving the entire body, with violent convulsions and loss of consciousness). Alternatively, an epilepsy condition may arise due to a known cause, known as symptomatic generalised (or partial) epilepsy. Symptomatic generalised epilepsy results from widespread damage to the brain, often during birth, leading to seizures, in addition to other neurological disorders such as mental retardation. Symptomatic partial epilepsy is the most common type of epilepsy which develops in adulthood, and is caused by localised damage to the brain, resulting from a stroke for example.

1.1.2 The hippocampus and seizure

Status epilepticus (SE) is a term given to describe when a seizure persists for a prolonged period of time, which in the past was considered to be over 20mins, but is more recently considered any seizure lasting longer than 5 minutes (reviewed in Chen & Wasterlain., 2006). Estimates of the incidence rate of SE, range from 6.8-40 per 100,000

people per year, with mortality associated with SE as high as 26% (Feen *et al*., 2008). Animal models of self-sustaining status epilepticus (SSSE), such as the perforant path stimulation (PPS) model, have revealed the areas of the brain metabolically up-regulated during SE. These areas include the hippocampus, amygdala, the substantia nigra, the nucleus accumbens, the caudate putamen and the medial thalamus (Wasterlain *et al*., 2002), with the hippocampus a major area of focus in epilepsy research.

The hippocampus is an important part of the limbic system, playing a major role in memory (Bliss & Collingridge., 1993) and spatial navigation (Bilkey & Clearwater., 2005). The hippocampus consists of six distinct regions: the hippocampus proper, subiculum, dentate gyrus (DG), presubiculum, parasubiculum and entorhinal cortex. The hippocampus proper can be further sub-divided into 4 fields (CA1-CA4), with CA4 considered to be part of the DG. Axons from the dentate granule cells, termed mossy fibres, spread out into the CA3 region (Blacksted *et al*., 1970), and form excitatory connections with both the proximal dendrites of CA3 principal cells and with inhibitory interneurons (Henze *et al*., 2000). SE is known to disrupt normal excitatory signalling from the mossy fibres, whilst also disturbing inhibitory interventions of the hippocampus. More specifically, SE stimulates excessive GABAergic signalling in the DG, resulting in γ-aminobutyric acid (GABA) release. The GABA receptors, once bound by GABA, internalise, effectively removing GABA inhibitory control from the hippocampus. This indirectly increases neuronal excitability, increasing the likelihood of a seizure (Sankar *et al*., 1997; Wasterlain *et al*., 1996; Walker *et al*., 2001). Furthermore, enhanced excitatory glutamate release is observed from the mossy fibres,

following activation of the dentate granule cells, leading to elevated neuronal firing (Gutierrez *et al.,* 2003).

The GABAergic and glutaminergic systems play a key role in epileptic seizure generation, and seizures are often attributed to an imbalance between the excitatory glutamate and the inhibitory GABA pathways (Tasker & Dudek., 1991). It has been shown that inhibition of the GABAergic pathways via $GABA_A$ receptor blockade, induces epileptiform activity (Collins *et al*., 1983), whilst pharmacological intervention to enhance GABAergic inhibition, are shown to prevent seizures in humans (Gale., 1992). Conversely, the glutamate receptor agonist, Kainate, is known to induce seizure-like characteristics and is routinely used in kainic acid (KA)-induced seizure models (reviewed in Fisher., 1989), whilst many ACDs act as glutamate receptor antagonists, such as felbamate, topiramate and Phenobarbital (Rogawski & Loscher., 2004a). It should be noted here, that throughout this thesis I will discuss findings from animal models, which whilst are not true seizures, do exhibit many of the characteristics. Therefore I will refer to such models as 'seizures', for brevity, throughout.

1.1.3 Molecular changes associated with epileptic seizure

Epileptic seizures induced complex biochemical and pathophysiological changes within the brain. These changes can occur very quickly, and within the first few seconds of a seizure, neurotransmitter release is activated, specific ion channels are activated whilst others are deactivated, and receptor phosphorylation occurs. Following this, within minutes, receptor trafficking is initiated, with the trafficking of GABA and glutamate receptors shown to be responsible for key cellular adaptations. In the longer

time period of a few hours, neuropeptide expression in altered, with enhanced pro-convulsant neuropeptide expression and decreased anti-convulsant neuropeptides observed, further enhancing neuronal excitability (Wasterlain & Chen., 2008).

It has been shown that during the transition from a single seizure into SSSE, $GABA_A$ receptors translocate from the neuronal membrane, into the cytoplasm, reducing the overall number of receptors available at the synapse to bind to the inhibitory GABA (Naylor *et al.,* 2005). In addition, it has been shown that the quantity of $GABA_A$ receptor γ_2 and $\beta_{2\text{-}3}$ subunits at the synaptic membrane also diminish following SE (Naylor & Wasterlain., 2005; Naylor *et al*., 2005). Furthermore, AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartic acid) subunits move to the synaptic membrane resulting in neuronal hyperexcitability (Mazarati & Wasterlain, 1999; Wasterlain *et al*., 2002b). These systems are of great significance to SSSE, with NMDA receptor antagonists shown to abolish the maintenance phase of SSSE in rats (Mazarati & Wasterlain., 1999).

Seizures have also been shown to stimulate the expression of a diverse range of genes, including immediate-early genes such as the transcription factors (TFs) c-fos and c-jun (Morgan & Curran., 1991; Kiessling & Gass., 1993), proteases such as the extracellular serine protease tPA (tissue-plasminogen activator) (Qian *et al*., 1993), the membrane trafficking protein synaptotagmin IV (Glisovic *et al*., 2007), and microRNAs, such as the CREB-regulated microRNA miR-132 (Nudelman *et al.,* 2009). In addition, changes in ion channel expression have been observed in KA seizure models, including an up-regulation of both sodium channel Nav1.6 (Blumenfeld *et al*., 2009) and voltage-dependent calcium channel, β2 subunit (CACNB2) expression (Gastaldi *et al*., 1998)

and a suppression of HCN2 (Hyperpolarization activated cyclic nucleotide-gated potassium channel 2) expression (Powell *et al*., 2008). Finally, certain ligand-gated receptors, such as the tachykinin receptor NK3R, have been shown to be elevated following KA-induced seizures (Roder *et al*., 1994).

The brain derived neurotrophic factor, BDNF, is also considered to play a key role in seizure and epileptogenesis, with high levels of BDNF, and its receptor, trkB found in brain areas associated with seizure susceptibility, such as the hippocampus. The view that BDNF is important in epileptogenesis has arisen from reports employing the kindling model. In this model, repeated, focal application of initially subconvulsive electrical stimuli is given, until eventually an intense focal and tonic–clonic seizure is generated. Expression of both BDNF and trkB are greatly up-regulated in kindling and other seizure models (Isackson *et al*., 1991; Mudo *et al*., 1996; Bengzon *et al*., 1993; Poulsen *et al*., 2002). Kokaia *et al,* reported a greater than 2x fold reduction in the rate of kindling development in BDNF heterozygous (+/-) mice (Kokaia *et al*.1995), whilst transgenic mice over-expressing BDNF have been shown to suffer more severe seizures in response to KA (Croll *et al.,* 1999). Furthermore, conditional deletion of trkB, has been shown to prevent epileptogenesis (He *et al*., 2004).

1.1.4 Neuropeptides in epilepsy

As aforementioned, the hippocampus is considered to be a key brain area in epilepsy, where numerous neuropeptides are expressed. These include the tachykinins Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), the cocaine and amphetamine-regulated transcript (CART), calcitonin gene related peptide (CGRP),

galanin and neuropeptide Y (NPY) (Sperk *et al*., 1990., Mathe, 1999; Schwarzer *et al*., 1995; Freund *et al*., 1997; Gruber *et al*., 1994; Seress *et al*., 2004; Baraban., 2004). SSSE is associated with a change in expression of many of these neuropeptides, including a reduction of the inhibitory neuropeptides galanin (Mazarati *et al*., 1998), dynorphin (Mazarati *et al*., 1999), somatostatin (Sperk *et al*., 1986) and NPY (Gruber *et al.,* 1994). Galanin is known to be a potent inhibitor of SSSE, with galanin over-expressing mice exhibiting enhanced resistance to the development of SSSE (Mazarati *et al*., 2000).

In addition, SSSE is associated with the increased expression of the proconvulsant tachykinins, SP (Liu *et al.,* 1999; Liu *et al*., 2000) and NKB in the hippocampus (Sperk *et al*., 1990; Marksteiner *et al*., 1992; Chen *et al*., 2008). SP and NKA are encoded by the same gene, the tachykinin 1 gene (TAC1), also termed pre-protachykinin A (PPT-A), with elevated TAC1 expression following KA-induced seizure, corresponding to enhanced expression of NKA in the rodent hippocampus following electoconvulsive stimulation (Stenfors *et al*., 1992). Furthermore, both SP and NKB have been shown to increase the frequency, and in some cases, the duration of epileptiform discharge in the entorhinal cortex (Maubech *et al*., 1998). The deregulation of inhibitory excitatory neuropeptide expression following an insult may impact upon the outcomes of the epilepsy condition, such as seizure duration and neuronal damage.

1.1.5 Epileptogenesis

The term ‘Epileptogenesis’ refers to a phenomenon in which the brain undergoes molecular and cellular alterations following a brain-damaging insult (e.g., traumatic

brain injury or stroke). These modifications increase neuronal excitability and eventually lead to the occurrence of recurrent spontaneous seizures, i.e. epilepsy (Pitkanen *et al*., 2007). Cellular alterations include neurogenesis, neurodegeneration, axonal sprouting, dendritic remodelling, angiogenesis and acquired channelopathies (Pitkanen *et al*., 2009). How this process occurs is poorly understood, however alterations in gene expression and neuronal reorganization are two proposed factors in epileptogenesis. Seizures are known to induce dramatic alteration of gene expression profiles and these changes can bring about long-term structural and functional changes within the brain. A latent period exists between the insult and the development of the epilepsy condition proper, and such structural and functional changes are proposed to occur during this latent period. This latent period can differ depending on the initial insult, for example epilepsy (spontaneous seizures) take up to 1 month following an initial seizure or SE, whilst the latency period following traumatic brain injury can be up to several months (Pitkanen *et al*., 2007).

It is known that during epileptogenesis, several cellular alterations occur including neuogenesis and neurodegeneration. In animal models, it has been shown that neurogenesis can be activated in the DG for several weeks following SE, contributing to aberrant neuronal network reorganisation (Parent *et al*., 2007; Parent *et al*., 1997). Furthermore, in the epileptic rodent brain, migration of newly born neurons can be disturbed by seizures, resulting in their ectopic location in the hilus, aberrant connectivity, and as a consequence, enhanced excitability (Scharfman *et al*., 2000). In terms of structural alterations, there is a well-established reorganization of the axons of the dentate granule cells (Proper *et al*., 2000), the CA1 pyramidal neurons (Esclapez *et*

al., 1997) and the interneurons (Magloczky *et al*., 2000), in a process known as 'sprouting'. In fact the sprouting of glutamatergic granule cell axons or 'mossy fibres', is one of the most widely studied forms of axonal plasticity in epilepsy. In addition, there is evidence pointing towards the stimulation of angiogenesis, with the increased expression of angiogenic factors following SE observed (Hunsberger *et al*., 2005).

1.1.6 Epigenetics and epileptogenesis

In addition to changes in gene expression and structural reorganization within the brain, there is now increasing evidence to support a role for epigenetic changes during epileptogenesis. The term 'epigenetics' refers to changes in a gene, which occur without affecting the DNA sequence, with chromatin modification being a prime example. Examples of modifications include DNA methylation, or changes to histone methylation or acetylation status. Histone modifications have been reported following both electroconvulsive seizures and KA-induced SE, at the promoters of numerous genes, including the TFs c-fos and CREB, BDNF and the glutamate receptor GluR2 (Huang *et al*., 2002; Tsankova *et al*., 2004; Taniura *et al*., 2006). SE-induced H4 acetylation of the BDNF P2 promoter was found to correlate with elevated BDNF mRNA expression (Tsankova *et al*., 2004), whilst deacetylation of histones at the GluR2 promoter was found to induce reduced GluR2 expression (Sanchez *et al*., 2001; Jia *et al*., 2006).

Support for the importance of epigenetic changes during epileptogenesis comes from the ACD valproic acid (VPA), which is a HDAC (histone deacetylase) inhibitor. KA-induced seizures have been shown to stimulate neurogenesis in the DG (Parent *et*

al., 2007; Parent *et al*., 1997) and VPA is known to potently block seizure-induced neurogenesis, through HDAC inhibition, modulating HDAC-dependent gene expression within the DG (Jessberger *et al*., 2007). Furthermore, the neuron restrictive silencing transcription factor (NRSF), also termed repressor element-1 silencing transcription factor (REST), which has a well documented role in coordinating epigenetic change, via the recruitment of a battery of co-repressors and chromatin remodelling enzymes (reviewed in Ooi & Wood., 2007), has been shown to be up-regulated in rodent models of SSSE (Palm *et al*., 1998; Spencer *et al*., 2006). In this thesis, the importance of such chromatin remodelling TFs will be explored, with regards to pro-convulsant gene expression regulation, and as potential targets for ACD action. Our principle hypothesis is that chromatin remodelling or epigenetics, is a fundamental mechanism underlying the progression from a single seizure, into the condition of spontaneous seizures, i.e. underlying epileptogenesis.

1.1.7 Seizure Models

An important aspect in testing the theory that epigenetics and gene regulation have a key role to play in epileptogenesis, is the availability of appropriate models. To date, the majority of models employed in epilepsy research are *in vivo* animal models. These include the induction of SE via either electrical stimulation, such as the PPS model, or through chemoconvulsants, such as pilocarpine or KA administration. These models are believed to reflect some of the described molecular and cellular changes which occur during epileptogenesis. For example, PPS has been shown to activate the DG granule cells, invoking excessive glutamate release (Mazarati *et al.,*1998), whilst pilocarpine has been shown to activate the cholinergic muscarinic receptors, in turn

modulating GABAnergic inhibitory signalling pathways (Kumar & Buckmaster., 2006). Whilst such models are excellent tools for studying gene expression changes during and post SE, it is often important to begin investigations using a cellular model so that we can address the biochemistry and cellular pathways in a more homogenous cell population where one can more easily control the challenge.

There are two main types of cellular models one may wish to utilise, these being immortalised cell lines or primary cell cultures. Our group has previously employed the latter option, to reveal that isoforms of the TF NRSF are modulated in terms of sub-cellular localisation in response to KA, in dissociated rat hippocampal cultures (Spencer *et al*., 2006). Such an approach has the benefits that one can experiment on regions that are critical in seizure progression, such as hippocampi, however the number of cells one can obtain from a specific tissue from a sacrificed animal, is often too few to perform certain experiments such as chromatin immunoprecipitation (ChIP). Furthermore the heterogeneity still does not allow for addressing cellular changes in a specific subpopulation of cells in the hippocampus and can present problems for certain biochemical and molecular techniques. Due to these reasons, this thesis will utilise immortalised cell lines throughout.

Despite decades of experimental research into epilepsy and seizure, there is yet to be a single cell line deemed to be the seizure-model cell line. This is perhaps unsurprising given the complexity and heterogeneity of the cells affected within an epileptic brain. However, it was important to establish a useful cell line model for use throughout this thesis. Numerous neuronal cell lines were available for experimentation, including the rodent PC12 cell line, which are derived from rat adrenal medulla (Greene

& Tischler., 1976), and have previously been used by our group to explore the activity of the SP-encoding TAC1 gene promoter (Paterson *et al*, 1995). For this thesis, a human cell line was preferred, and two human neuroblastoma cell lines have been utilised: SH-SY5Y and SK-N-AS.

The human SH-SY5Y cell line is derived from the SK-N-SH cell line, first isolated in 1970, and have been shown to exhibit a number of neuronal features, including dopamine beta hydroxylase activity (Biedler *et al*., 1978), an enzyme linked to seizure susceptibility (Warter *et al.,* 1975; Szot *et al.,* 1999). This cell line has been shown to be responsive both to glutamate and NMDA (Sun *et al*., 1998; Jamsa *et al.,* 2006), suggesting that these cells may endogenously express glutamate receptors, and indeed Nair *et al* have shown that SH-SY5Y cells do express the NMDA receptor (Nair *et al*., 1996). Furthermore, recent findings have shown that this cell line is also responsive to KA stimulation (Cannarsa *et al*., 2008), indicating that this cell line may be a good cell model to investigate molecular changes in response to aberrant glutaminergic signalling, characteristic of seizure. In support of this, SH-SY5Y cells have been shown to express a number of neuronal genes implicated in epilepsy, including the BDNF receptor TrkB (Ehrhard *et al*., 1993), the Kv3 potassium channel (Friederich *et al*., 2002) and the cholinergic receptor nicotinic alpha 4 nAChR (Liu *et al.,* 2006).

The second neuroblastoma cell line employed in this thesis, the SK-N-AS cell line, was first isolated in 1981, and has received less attention than the SH-SY5Y cell line. Early publications revealed that SK-N-AS cells synthesis Insulin-like growth factor II (IGF-II) and express its receptor (El-Badry *et al*., 1989). IGF-II has been showed to be

expressed within the hippocampus, and is thought to have an important role in neural growth and differentiation (Dore *et al*., 1997). Interestingly, both IGF-I and IGF-II have been shown to be reduced in the hippocampus in both rodent kindling models (Kalynchuk *et al*., 2002), and following KA administration (Kar *et al*., 1997), suggesting that SK-N-AS cells may also be responsive to chemoconvulsant stresses, and therefore may be of use as a cell line model to explore molecular changes in response to pro-convulsant challenges. Consistent with this, preliminary findings from our group have revealed that this cell line endogenously expresses the pro-convulsant neuropeptides SP and NKB, as well as the TF NRSF, which as discussed earlier are correlated with seizure.

Throughout this thesis, the SH-SY5Y and SK-N-AS cell lines will be employed to explore molecular events relevant to seizure. Such cell lines are useful in initial experimentation, to build hypotheses and unlock questions, but it is important to stress that such models cannot reflect the true diversity nor the complexity of events which occur throughout the brain in response to seizure. As such, this thesis aims to open up lines of research for future *in vivo* experimentation.

1.2 The Tachykinins

1.2.1 The Tachykinin Family

The tachykinins are a group of peptides which function as excitatory neurotransmitters, sharing a common C-terminal amino acid sequence (Phe-X-Gly-Leu-Met-NH_2) (reviewed in Severini *et al*., 2002). They are widely distributed within the central nervous system (CNS) and the peripheral nervous system (PNS), with some recent findings in non-neuronal tissue (Pennefather *et al*., 2004). Since the early discovery of SP in the 1930's (von Euler & Gaddum., 1931), a number of other tachykinin family members have been discovered, including NKA (Kangawa *et al*., 1983; Nawa *et al*., 1984), NKB (Kanazawa *et al*., 1984; Kimura *et al*., 1984), neuropeptide K (NPK) (Tatemoto *et al*., 1985), neuropeptide γ (NPγ) (Kage *et al*., 1988) and most recently hemokinin-1 (HK-1) (Zhang *et al*., 2000). Tachykinins are predominately synthesised in the neurons of the CNS and the PNS and are stored in dense core vesicles (McCarthy & Lawson., 1989; Floor *et al*., 1982). The tachykinin family members are implicated in a wide range of processes and disorders such as neurotransmission (Otsuka & Yoshioka., 1993; Patacchini *et al*., 1998), pain (Duggan *et al*., 1987; Duggan *et al*., 1988; Cao *et al*., 1998), cognitive and psychiatric disorders (Kramer *et al*., 1998; Maubach *et al*., 1999), epilepsy (Liu *et al*, 1999; Wasterlain *et al*, 2000), Parkinson's disease (Barker., 1991; Chen *et al*, 2004), Huntington's disease (HD) (Richfield *et al*., 2002), pre-eclampsia (Page *et al*., 2000; Page *et al*., 2001; Page *et al*., 2006) and breast cancer (Patel *et al*., 2005; Rao *et al*., 2004; Corcoran *et al*., 2008).

Tachykinins interact with specific membrane bound G protein-coupled receptors (GPCRs), with three distinct tachykinin receptors discovered to date: NK1R, NK2R and NK3R (Gerard *et al*., 1991; Takeda *et al*., 1991; Gerard *et al*., 1990; Buell *et al*., 1992). These tachykinin receptors belong to the rhodopsin-like family of GPCRs, sharing a common structural motif consisting of bundle of seven transmembrane domains, three extracellular loops, three intracellular loops, an extracellular amino-terminus and a cytoplasmic carboxy-terminus. The tachykinins SP, NKA and NKB act as agonists on all three tachykinin receptors, whilst also exhibiting preferential binding to NK1R, NK2R and NK3R, respectively. (Mussap *et al*., 1993; Regoli *et al*., 1994; Maggi, 2000). The expression of these receptors, has been shown to match that of the high affinity tachykinins, with NK1R and NK3R observed in the CNS and peripheral tissue and NK2R found in the peripheral tissue only (reviewed in Pennefather *et al*., 2004).

1.2.2 Neurokinin B (NKB)

NKB is encoded by the preprotachykinin-B (PPT-B) gene, also referred to as TAC3 (human) (Page *et al.,* 2000) or TAC2 (rodent) (Chawla *et al.,* 1997). The human TAC3 gene consists of seven exons, with exon 5 encoding the NKB peptide (Figure 1.1a). Like the other tachykinins, NKB is proposed to act as an excitatory neurotransmitter, and has been shown to be expressed throughout the CNS (Lucas *et al*., 1992; Moussaoui *et al*., 1992), and the PNS, with NKB and its endogenous high affinity receptor (NK3R) shown to be expressed in human airways and pulmonary veins and arteries. These findings suggest an addition role for NKB in lung pathophysiology (Pinto *et al*., 2004). NKB mRNA has also been recorded in human lymphocytes, monocytes, neutrophils and eosinophils, suggesting NKB plays a role in the

pathophysiology of the inflammatory process (Klassert *et al*., 2008). Furthermore, NKB expression has been demonstrated in the placenta (Page *et al*., 2000) and the myometrium (Patak *et al*., 2003), and NKB is implicated in pre-eclampsia, with elevated NKB expression found in pre-eclamptic placenta (Page *et al*., 2006). In addition, recent findings have correlated mutations of both the NKB and NK3R genes, with hypogonadotropic hypogonadism, suggesting a key role for NKB in the control of reproduction (Topaloglu *et al*., 2009). NKB therefore, appears to have several important roles throughout the body, in addition to neurotransmission.

The related tachykinin, SP, has been shown to be of importance in epilepsy, with elevated levels of SP observed in rodent models of SSSE (Wasterlain *et al.,* 2000). NKB has also been implicated in epilepsy, with elevated NKB immunoreactivity and mRNA levels observed in rat hippocampus following KA administration (Marksteiner *et al*., 1992b). In support of this, NK3R, has been shown to exacerbate KA induced neuronal death in mice (Chen *et al*., 2008), and the NK3R agonist senktide, induces hyperlocomotor activity in rodents (Nordquist *et al*., 2008). Furthermore, SP and NKB have been shown to increase the frequency, and in some cases, the duration of epileptiform discharge in the entorhinal cortex (Maubach *et al*., 1998). Interestingly, NKB has recently been shown to be up-regulated in a rodent model of Parkinson's disease, following administration of the dopamine mimic L-DOPA, whilst activation of the NK3R, evokes dopamine release (Zhang *et al*., 2008), indicating a role for NKB in the dopaminergic system. Subsequently, due to the importance of NKB in a multitude of conditions including epilepsy, pre-eclampsia and lung pathophysiology, it is of great importance to gain a greater understanding of the regulation of the NKB gene.

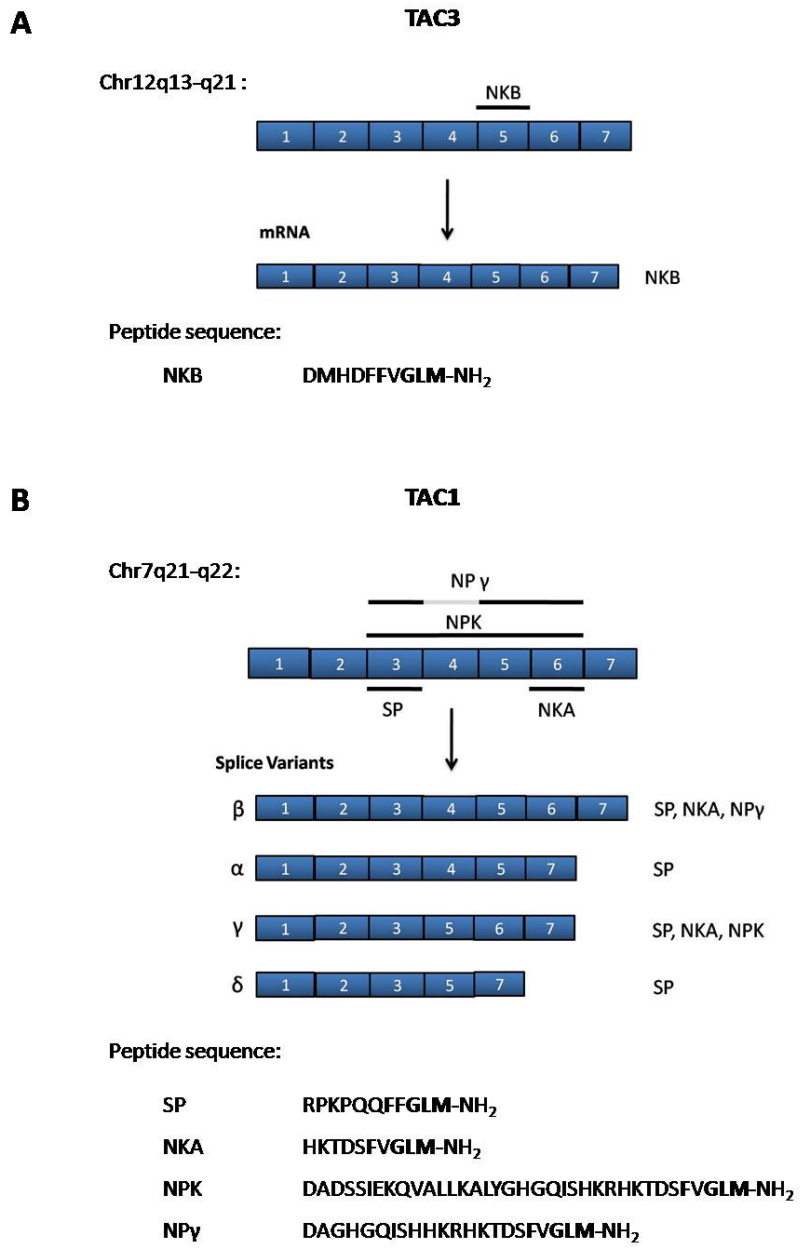

Figure 1.1 Schematic representation of the alternative splicing which results in the synthesis of different neuropeptide. (A) NKB is derived from the TAC3 gene, with exon 5 encoding the neuropeptide. (B) The human TAC1 gene encodes four neuropeptides: SP, NKA, NPK and NPγ. Chromosomal position of genes are given, as are the resulting peptide sequences, with the common sequence highlighted.

1.2.3 Regulation of NKB

The regulation of the NKB-encoding TAC3 gene is poorly understood, in comparison to that of the TAC1 gene. The only published findings are from Pal *et al*, who revealed that the rodent NKB gene (TAC2) is regulated by the GATA family of TFs, in non-neuronal cells. GATA-1 was shown to activate TAC2 transcription in erythroid cells, by replacing GATA-2 from a GATA motif located in intron 7 (Pal *et al*., 2004). However, NKB regulation in human or indeed any neuronal cells has yet to be determined.

Potential candidates have arisen based on processes and systems which modify NKB expression. For example, NKB expression is found to be enhanced in the arcuate nucleus in post-menopausal women, i.e. those with low oestrogen, whilst oestrogen replacement suppresses NKB expression, suggesting that oestrogen represses NKB expression. This has been shown to be mediated by the oestrogen receptor (ERalpha) (Dellovade & Merchenthaler., 2004). The upstream stimulatory factor (USF) 1 (USF1), which has been correlated with seizures (Sirito *et al*., 1998), has also been shown to be activated by the ERalpha signalling pathway, inducing USF1 binding to the cathepsin D promoter (deGraffenried *et al*., 2004). Thus a case may be made for USF1 binding to the NKB promoter in a similar manner. Support for such a hypothesis comes from the fact I have located a putative E box motif, a DNA sequence to which USF and other basic-Helix-Loop-Helix (bHLH) factors recognise and bind to, within the TAC3 proximal promoter (Figure 1.2). Furthermore, NKB has been shown to co-localise with arginine vasopressin (AVP), in AVP expressing neurons (Hatae *et al*., 2001). AVP is also regulated by USF factors binding to an E box within the AVP promoter (Coulson *et al*.,

1999; Coulson *et al*., 2003) and I propose that USF may regulate the NKB promoter in a similar manner.

Another TF, shown to regulate TAC1 expression, NRSF, has also been shown to regulate AVP expression (Quinn *et al*., 2002). Consequently, NRSF is a candidate regulator of NKB expression, due to its existing regulation of neuropeptides, and as NRSF has been shown to be up-regulated following seizure (Palm *et al*., 1998; Spencer *et al*., 2006), matching the changes observed for NKB (Sperk *et al*., 1990; Marksteiner *et al*., 1992b). Support for a role of NRSF in NKB regulation came from a recent bioinformatics search, which identified a putative NRSF recognition site (NRSE, or the neuron restrictive silencing element), upstream from the rodent TAC2 gene (Otto *et al*., 2007). This site was however some 20kb upstream from the gene, and I have since identified an alternative putative NRSE much closer to the transcription start site (TSS), spanning +55 to +71 of the human TAC3 promoter (Figure 1.2). This putative NRSE was identified based on the NRSE consensus sequence (Figure 1.6a). Furthermore, NKB expression in striatal neurons has been shown to be distributed in association with μ-opioid receptor localisation (Furuta & Kaneko., 2006), with the μ-opioid receptor also known to be regulated by NRSF (Formisano *et al*., 2007). In this thesis, the regulation of the human NKB gene will be explored, focusing on the potential regulatory roles of both USF and NRSF TFs in governing NKB promoter activity and endogenous NKB expression.

Figure 1.2 Sequence of the human NKB (TAC3) proximal promoter spanning -757 to + 181. Putative NRSE and E box motifs are highlighted in yellow and green respectively. The putative NRSE spans from +50 to +71, and the putative E box spans from +160 to +166.

1.2.4 Substance P (SP)

Perhaps the most characterised tachykinin is SP, which is encoded by the TAC1 gene. Like other tachykinins, SP is widely distributed throughout the CNS and the PNS and is predominantly synthesised within neurons and stored in dense secretory vesicles. Upon cellular excitation, SP is released and acts upon appropriate receptors, including its high affinity NK1R, to evoke a variety of responses (Maggi, 1993; Pennefather *et al.*, 2004). Due to its role in neuronal communication, SP is classically considered to be a

neurotransmitter, but recent findings have implicated other roles for SP, including a role as an inflammatory molecule, analogous to a cytokine (Pascual *et al*, 1992; Luger & Lotti, 1998). SP is implicated in a wide range of disorders including Parkinson's disease (Barker., 1991; Chen *et al*, 2004), HD (Richfield *et al*., 2002), pain (Duggan *et al*., 1987; Duggan *et al*., 1988), cognitive and psychiatric disorders (Kramer *et al*., 1998; Maubach *et al*., 1999) and breast cancer (Patel *et al*., 2005; Rao *et al*., 2004; Corcoran *et al*., 2008).

SP is of particular interest to this thesis based upon its pro-convulsant role. Like NKB, SP is up-regulated in the hippocampus following PPS-induced SSSE (Liu *et al*,. 1999; Wasterlain *et al*., 2000). In addition, studies have shown that hippocampal administration of SP can induce SSSE, at electrical stimulation below the normal SSSE-inducing threshold (Liu *et al*., 1999). Furthermore, elevated SP expression in the hippocampal CA3, CA1 and DG regions have been implicated in neuronal damage following SSSE induced by either PPS or KA (Brene *et al*., 1992; Liu *et al*., 1999a; Liu *et al*., 1999b; Liu *et al*., 2000). The findings that NKA, an alternative peptide encoded by the TAC1 gene, is also up-regulated during KA treatment (Sperk *et al*., 1990), suggests that seizure has a direct affect upon the TAC1 gene, and that TAC1 regulation is of upmost importance in the early stages of SSSE. In support of this, TAC1 knockout mice show reduced seizure duration and severity in response to KA treatment (Liu *et al*, 1999b). Finally, the SP peptide itself is shown to contribute to limbic seizure activity, as antagonists to NK1R, have been shown to prevent KA-induced SSSE (Zachrisson *et al*., 1998).

1.2.5 Regulation of TAC1

Unlike NKB regulation, the regulation of the TAC1 gene is relatively well documented. The human TAC1 gene is 8kb long and consists of seven exons which give rise to four alternatively spliced mRNA transcripts, designated α, β, γ and δ (Nawa *et al*, 1984; Carter & Krause, 1990). These transcripts differ in their exon combinations, for example αTAC1 lacks exon 6 whereas γTAC1 lacks exon 4. Four neuropeptides arise from these transcripts: SP, NKA, NKP and NPγ, with the SP precursor synthesised from all four isoforms (as summarised in Fig.1.2b) (Macdonald *et al*, 1988). Translation of mature TAC1 mRNA generates a large polypeptide which is transported to the Golgi apparatus, where it is cleaved and the active peptide is then packed into secretory granules destined for the nerve endings.

Studies into the regulation of the TAC1 gene have predominantly focused upon the rat TAC1 proximal promoter sequence, although some studies has been undertaken exploring human TAC1 gene regulation (Mackenzie *et al*., 2000; Mackenzie & Quinn., 2002; Greco *et al*., 2007; Murthy *et al*., 2008; Reddy *et al*., 2009). Early DNase 1 footprinting studies revealed a number of *cis*-acting regulatory elements within the rat TAC1 proximal promoter, including a TATA box spanning -22 to -28 in relation to the TSS (Carter & Krause., 1990), initiator elements located -20 to +4 (Mendelson., 1995), an octamer-binding motif, Sp-1 motifs, activation protein 1/cAMP response element (AP1/CRE) motifs, multiple E box sites and an unidentified but distinct factor (Fiskerstrand and Quinn, 1996; Fiskerstrand *et al*., 1999; Fiskerstrand *et al*., 2000; Fiskerstrand *et al*., 1997; Mendelson and Quinn, 1995; Paterson *et al*., 1995a; Paterson, *et al*., 1995b; Paterson, *et al*., 1995c; Mendelson *et al*., 1998). A summary of

the known or proposed TF binding sites, within the rodent TAC1 proximal promoter, is given in Figure 1.3. Subsequently, sequence comparisons between TAC1 and AVP, revealed a domain with high sequence homology to the NRSE located within the proximal promoter, suggesting that NRSF maybe be a candidate regulator of TAC1 (Quinn *et al*., 2002). In support of this, endogeneous levels of NRSF and its truncated isoform were reported to be elevated following KA-induced SE (Palm *et al*., 1998), consistent with an increase in TAC1 (Wasterlain *et al*., 2000). Our lab has recently shown that both NRSF and a truncated isoform can modulate TAC1 expression, as over-expression of these TFs led to both an increase in TAC1 promoter activity, and also an increase in endogenous TAC1 expression in dissociated rat hippocampal cultures (Spencer *et al*., 2006). Since these findings, a number of other groups in addition to our own, have shown that NRSF regulation of TAC1 is cell-specific, with both repression and activation of the TAC1 promoter or endogenous expression, governed by NRSF in a tissue-dependent manner (Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009).

The USF family are also potential regulators of TAC1 expression, based upon the discovery of multiple E-box motifs within the rat TAC1 proximal promoter. The E box canonical sequence CANNTG is recognised by the bHLH family of proteins, to which the ubiquitously expressed USF belongs. One particular E-box motif, spanning nucleotides -67 to -47 (Paterson *et al*., 1995) was shown to be functional, regulating the response of the rat TAC1 promoter to nerve growth factor (NGF) stimulation in rat dorsal root ganglion (DRG) (Gerrard *et al*., 2005). Another *cis*-regulatory element identified in the earlier studies, AP1/CRE, centered at -196, was also later shown to be functional, coordinating forskolin-induced TAC1 promoter activity, in a PKA-dependent

manner (Calin-Jageman *et al*., 2006). More recently, a role for NFκB in the regulation of human TAC1 has been proposed. In non-neuronal cells, NRSF has been shown to bind to TAC1 and suppress its activation. Following stimulation of mesenchymal stem cells (MSCs) with IL-1α, NRSF binding to TAC1 was found to decrease and suppression was alleviated. NFκB was also found to bind to the human TAC1 proximal promoter, and this binding was also diminished following IL-1α stimulation. Subsequently, NFκB was proposed to work in synergy with NRSF to bring about TAC1 suppression in non-neuronal cells (Greco *et al*., 2007). Furthermore, the chemokine Stromal-derived factor-1 alpha (SDF-1α) has been shown to modulate TAC1 expression, through the activation of NFκB in non-tumorigenic human breast cells (Corcoran *et al*., 2008).

In this thesis, I will explore the regulation of the rat (and in part the human) TAC1 promoter further, by investigating a possible synergistic regulation of the TAC1 promoter by the NRSF and USF TF families, akin to synergy proposed between NFkB and NRSF (Greco *et al*., 2007).

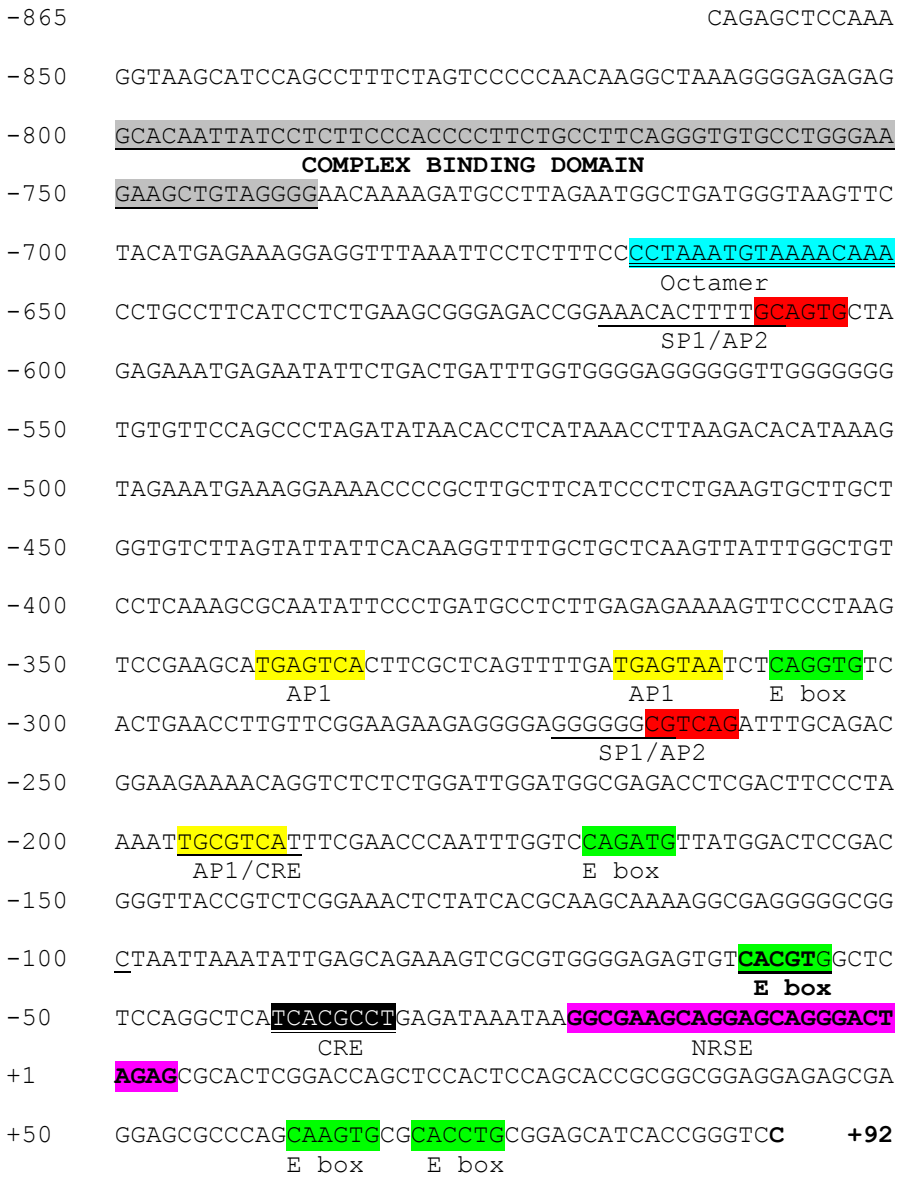

Figure 1.3 Sequence of the rat TAC1 proximal promoter region spanning -865 to +92. *Cis*-regulatory domains are highlighted as follows: Light green are E box motifs, pink is the NRSE, yellow is an AP1 element, with overlapping CRE site underlined, whilst separate CRE elements are highlighted in black. Red indicates an AP2 site, with overlapping SP1 sites underlined, light blue represents the octamer site, and the grey indicates a complex binding domain, which can bind a number of single or double stranded binding proteins.

1.3 Gene regulation

1.3.1 Transcription

The exquisite control of gene expression can occur through multiple regulatory mechanisms including translation, post-translational modifications and degradation. One of the earliest and most efficient control points is at the level of transcription, the synthesis of RNA from a DNA template. The transcription process is coordinated by the enzyme RNA polymerase (Pol), with eukaryotes having three nuclear enzymes Pol I-III, of which Pol-II is vital to the transcription of mRNA. Pol-II alone, is incapable of specific initiation and transcription, instead it requires the activity of the general transcription factors (GTFs), denoted TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, to assemble at the core promoter. Once assembled onto the core promoter, the GTFs form stable nucleoprotein complexes and recruits Pol-II, to form the pre-initiation complex (PIC). More specifically, the TATA-binding protein (TBP) in a complex with TAFs (TBP Associated Factors), collectively known as TFIID, first binds to the core promoter, which nucleates the formation of PIC (Pugh & Tjian, 1991). TFIIA and TFIIB subsequently bind to the TFIID complex, stabilising its interaction with the DNA. To this binds TFIIF, Pol-II, TFIIE and finally TFIIH, forming the basal transcription machinery. At this point, Pol-II and the GTFs are bound to the core promoter in an inactive state. The GTFs have a key role in the recognition of promoter sequences and to govern the response to regulatory factors, such as TFs. The basal transcription machinery consists of one final factor, known as Mediator. Mediator facilitates communication between regulatory proteins (i.e. TFs) and the transcriptional machinery, transducing regulatory information to Pol-II (Kelleher *et al*., 1990; Kim *et al*., 1994).

Once the PIC is established, a conformational change occurs in which 11-15bp of DNA surrounding the TSS are melted, and the template strand is positioned within the active site cleft of Pol-II (Wang *et al*., 1992). This melting step is mediated by the helicase activity of TFIIH (Tirode *et al*., 1999), which unwinds the DNA in an ATP-dependent manner (Holstege *et al*., 1997). Initiation of transcription begins with the phosphorylation of Pol-II at serine-5 by TFIIH, releasing Pol-II from the PIC, followed by phosphorylation of serine-2, which enables Pol-II to begin RNA synthesis in the elongation phase. The resulting transcript, known as pre-mRNA, undergoes further processing, including removable of introns via splicing, and the addition of a 5' cap (addition of modified guanine nucleotides to the 5' end) and a 3' poly-A-tail.

Pol-II transcription can be governed by three distinct classes of DNA sequences. The first class includes DNA sequences within the core promoter with perhaps the best characterised core promoter elements being the TATA box (consensus TATAAA) located approximately -25 from the TSS, and a pyrimidine-rich sequence near the TSS known as the initiator element (Inr). As A-T rich sequences require less energy to denature into single strands, it is thought the TATA box facilitates strand separation. Both the TATA box and the Inr are recognised by the GTFs to initiate transcription. The remaining two classes are both *cis*-regulatory domains: the proximal-promoter elements and the distal-promoter elements (enhancers/repressors) (see figure 1.4). TFs bind to these *cis*-regulatory domains, and affect transcription either directly, whereby they may affect the GTFs, inhibiting/enhancing PIC assembly, or indirectly, through chromatin remodelling, which will be discussed later. Examples of these include the transcriptional activator cAMP response element binding protein (CREB), which has been shown to

interact directly with TFIID, recruiting the coactivators CREB-binding protein (CBP) and p300, which facilitate recruitment of Pol-II to the promoter (Nakajima *et al*., 1997; Vo & Goodman., 2001).

Whilst the promoter region of many genes contains DNA sequences to which proteins can bind to, the mere presence of these DNA sequences, whilst necessary, is not sufficient for transcription factor binding. This is because DNA is packaged into chromatin, with DNA wrapped tightly around histone proteins. As histones affect protein-DNA interactions, and thus transcription, it is now widely accepted that chromatin has a significant role in coordinating transcriptional regulation of eukaryotic genes.

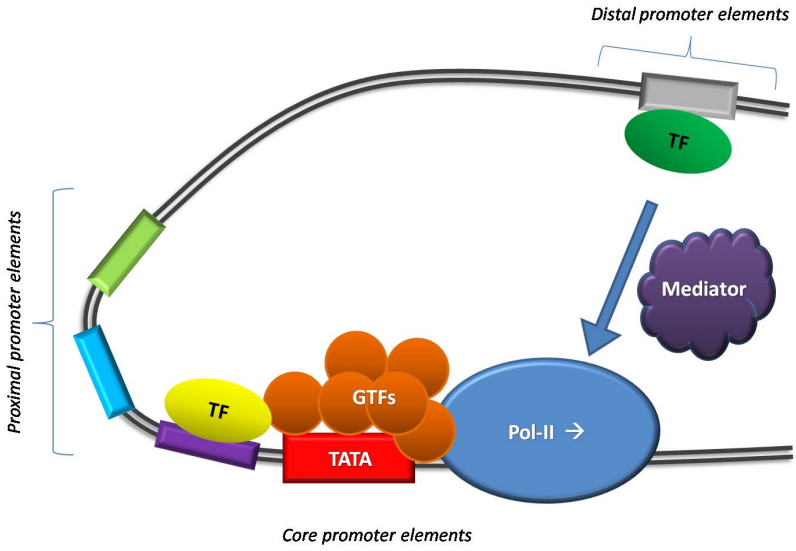

Figure 1.4 Schematic diagram to illustrate RNA polymerase and GTFs recruitment to the core promoter for transcription initiation. The three classes of DNA sequences are given, the core promoter (TATA box), the proximal promoter and the distal promoter elements. A single TF is shown to illustrate how they may modulate transcription, via direct association with the basal transcription machinery. Mediator is shown, which transducers regulatory information to Pol-II.

1.3.2 Chromatin

Approximately two metres of DNA is packaged within the nucleus of a cell, wrapped tightly around core histone proteins. The fundamental unit of chromatin is the nucleosome core particle which consists of 146bp of DNA wrapped 1.65 turns around a histone octomer, containing two central H3/H4 dimers, flanked by two H2A and H2B dimers (Arents *et al*., 1991). Histones are small proteins with protruding, charged, N-terminal tails, which extend out from the nucleosome between turns of DNA. As more than 20% of the histone amino acid sequence is positively charged (lysine and arginine), strong interactions with the negatively charged DNA backbone can occur. Furthermore, with more than 14 contact points between histone and DNA, the nucleosome complex has a high degree of stability under physiological conditions. However, under low ionic strength buffer conditions, chromatin decondenses to resemble 'beads on a string' (Oudet *et al*., 1975). At this level of compaction, chromatin is often referred to as 11nm fibres. Chromatin can become more compact, through the linking of nucleosomes by 'linker' histones, denoted 30nm fibres. It is believed that chromatin exists at an even greater level of compaction *in vivo*, and may exist as 80-100nm fibres (Belmont & Bruce., 1994).

The highly compact nature of chromatin inhibits many processes which require access to the DNA, including DNA repair, replication and transcription. An increasing appreciation of the role of chromatin in transcription has developed, following the discovery that nucleosomes impede transcription *in vitro* (Knezetic and Luse, 1986), whilst deletion of histones or their basic tails enhances gene expression *in vivo* (Han & Grunstein, 1988; Han *et al*., 1988). The highly packaged nature of the DNA can prevent

TFs from recognising and binding to target DNA sequences, and can impair the formation of PIC and binding of TBP to core promoter regions (Imbalzano *et al*., 1994). In order to activate gene expression, this highly compact structure needs to be unwrapped, and conversely, if gene expression needs to be silenced, then this compact structure can be regenerated. Two major mechanisms have arisen to meet these demands: Chromatin remodelling and histone modifications.

1.3.3 Chromatin remodelling

As I have mentioned, the structure of chromatin presents quite a formidable barrier to transcription. To overcome this, both activators and repressors utilise chromatin-remodelling enzymes to catalyse the movement of nucleosomes, altering the availability of DNA sequences to the basal transcription machinery. Due to the strength of the interactions between the negatively charged DNA and the positively charged histones, chromatin-remodelling enzymes require energy derived from ATP hydrolysis to drive DNA away from the nuclesome (Narlikar *et al*., 2001). Consequently, chromatin-remodelling complexes contain a central ATPase, and can be separated into three family groups based upon this central ATPase: the SWI/SNF family, the ISWI family and the CHD1 family. It was thought that chromatin-remodelling enzymes were associated with transcriptional activation, facilitating access of Pol-II to the core promoter, however a number of chromatin-remodelling enzymes have been shown to repress transcription. One example is BRG1, a member of the SWI/SNF family, which is recruited by NRSF, stabilising NRSF binding to target DNA sequences, enhancing repression of target genes (Ooi *et al*., 2006).

The consequence of chromatin remodelling include the unwrapping of DNA away from histone octomers, the formation of DNA loops, or the movement of nucleosomes to different translational positions, in a process known as 'sliding'. All of these result in a change in the accessibility of DNA to TFs (reviewed in Li *et al*., 2007). It has recently emerged that histone displacement can also occur, in which H2A/H2B dimers can easily exchange in and out of nucleosomes, and indeed entire histone octomers can be displaced in a process known as 'eviction'. Cooperation between TF binding, chromatin-remodelling complexes and actively transcribing Pol-II, can all mediate histone displacement (Adams and Workman, 1995; Bruno *et al*., 2003; Kireeva *et al*., 2002). Due to the fact that displaced histones can rebind to the same stretch of DNA, histone acceptors or chaperones (such as Asf1) are required to prevent this and are shown to be vital in histone eviction *in vivo* (reviewed in Li *et al*., 2007).

Following chromatin remodelling, a nucleosome–free region becomes available for the formation and binding of the PIC. During transcription elongation, histone acetyltransferase (HAT) complexes associate with the elongation machinery to acetylate histones in front of Pol-II, facilitating in the disassembly of downstream nucleosomes, onto awaiting histone chaperones. This can lead to histone loss, with partial loss of nucleosomes recorded over the coding regions of actively transcribed genes in yeast (Lee *et al*., 2004), and in *Drosophila* (Wirbelauer *et al*., 2005). Following the passage of Pol-II, the displaced histones are replaced or reassembled. This is coordinated by the histone chaperone Spt6, which colocalises with the elongating Pol-II (Kaplan *et al*., 2000). Spt6 interacts directly with displaced histones, enabling their assembly back behind the elongating Pol-II. Furthermore, histone H3 Lysine 36 (H3K36) is methylated

by the Pol-II bound Set2 methyltransferase, leading to a repressive environment behind the elongating Pol-II, preventing further PIC formation and subsequent transcription (summarised in Figure 1.5) (reviewed in Workman., 2006).

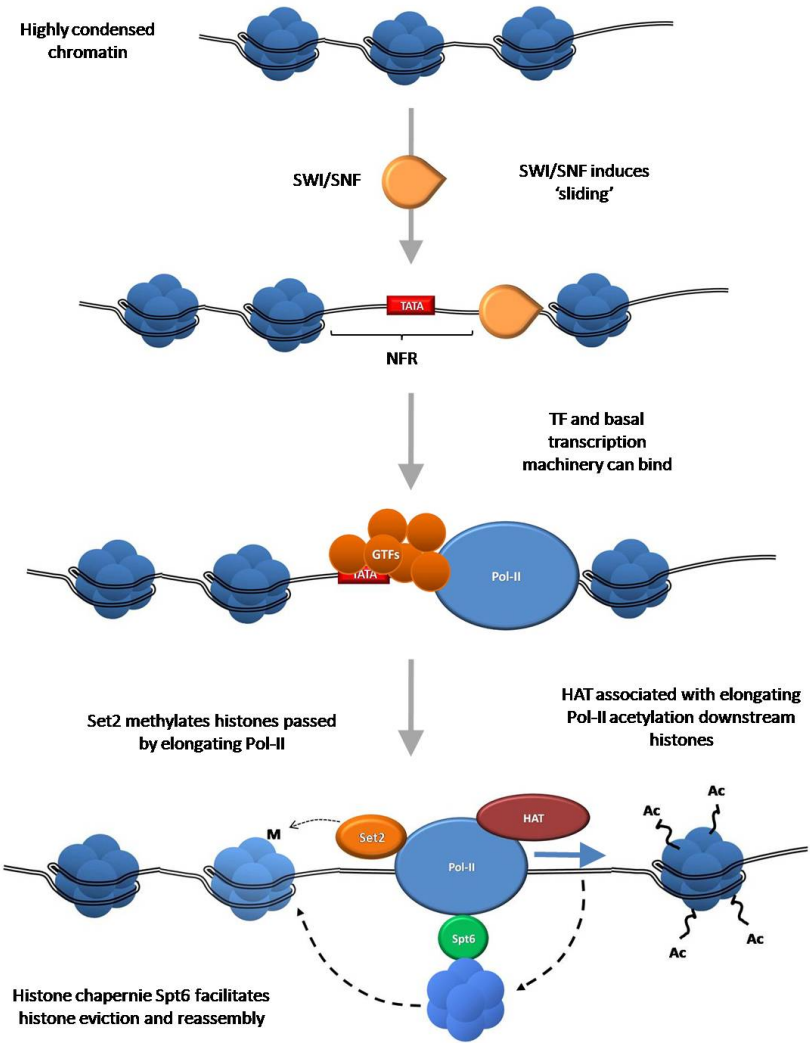

Figure 1.5 Schematic diagram illustrating chromatin remodelling during transcription. Condensed chromatin is unravelled by the SWI/SNF chromatin remodelling complex, sliding nucleosomes to generate a nucleosome free region (NFR), to which the basal transcriptional machinery can bind. Pol-II binds and initiates transcription, entering the elongation phase. During elongation, HATs associate with Pol-II to acetylate downstream histones, thus facilitating in the de-condensation of future chromatin. Histones can be displaced, and reassembled behind the elongating Pol-II, mediated by the histone chaperone Spt6. Finally, histones assembled behind the elongating Pol-II are methylated by the Set2 methyltransferase, resulting in a repressive environment behind Pol-II, preventing further PIC formation.

1.3.4 Histone modifications

Both the N-terminal tails and the globular domains of histones, are subject to a vast array of post-translational modifications, which include: methylation (of arginine (R) or lysine (K) residues); acetylation, ubiquitination, ADP-ribosylation, sumoylation, and serine/threonine phosphorylation (Reviewed in Kouzarides., 2007). To orchestrate these modifications, an array of enzymes has evolved which include HATs, HDACs, histone methyltransferases (HMTs), demethylases, ubiquitilases and serine/threonine kinases. Certain modifications are associated with active transcription, and are commonly referred to as euchromatin modifications, whilst modifications that are localised to inactive genes or regions, are often termed heterochromatin modifications (a summary of such modifications is given in Table 1.1). Most of the histone modifications known to date are localised in distinct regions, which may be upstream of the promoter, at the core promoter or at the 5' or 3' end of the open reading frame (ORF). The correct localisation of such modifications is vital in coordinating its affect on transcription. For example, methylation of H3K36 usually occurs within the ORF, and is often referred to as a hallmark of elongation (Guenther *et al*., 2007), however methylation of H3K36 at the promoter leads to transcriptional repression (Strahl *et al*., 2002). A similar finding has been observed following methylation of H3K9 (Vakoc *et al*., 2005) (Table 1.1).

Modification	Residues Modified	Active or Repressive
Acetylation	H3K56	**ACTIVE**
	H4K16	**ACTIVE**
	H3K9	**ACTIVE**
	H3K4	**ACTIVE**
Methylation	H3K4	**ACTIVE**
	H3K4	**ACTIVE**
	H3K79	**REPRESSIVE**
	H3K9	**REPRESSIVE/ ACTIVE**
	H3K27	**REPRESSIVE**
	H3K36	**REPRESSIVE / ACTIVE**
	H3K9	**REPRESSIVE / ACTIVE**
	H4K20	**REPRESSIVE**

Table 1.1. **Summary of histone modifications and their corresponding impact upon transcription**. Modified from Li *et al*., 2007.

1.3.4.1 Histone acetylation

Acetylation is perhaps one of the most well characterised modifications of histones, and is almost invariably associated with transcriptional activation and gene expression (Pokholok *et al*., 2005). It is believed that the acetylation of lysine residues neutralises the net positive charge of the histone, reducing the strength of the electrostatic interactions between the histone and the negatively charged DNA. This results in a more relaxed chromatin configuration, enhancing the accessibility of the DNA to binding proteins. The enzymes responsible for histone acetylation are the HATs, which can be separated into three families: GNAT, MYST and CBP/p300 (Sterner & Berger., 2000). The majority of acetylation sites are found within the N-terminal tails in a non-specific manner, although a number of more specific core histone lysines have since been found to be acetylated (see Table 1.1). This number has since risen to 18 histone acetylation sites following a genome wide study, with certain types

such as H3K9ac located at the TSS, and others such as H3K4ac, elevated in the promoter region (Wang *et al*., 2008).

Histone acetylation can also be reversed by the HDAC complexes which are correlated with transcriptional repression. There are three main families of HDACs: the class I and class II HDACs and the class III NAD-dependent enzymes of the Sir family. These enzymes remove acetyl-groups from lysine residues, without much specificity, although the yeast enzyme Sir2, has been found to display some selectivity for H4K16ac (Vaquero *et al*., 2006).

1.3.4.2 Histone methylation

In contrast to acetylation, methylation is a highly restricted modification, in terms of the specific residues targeted. The enzymes responsible for methylation, the HMTs, usually only modify a single lysine (or arginine) on a single histone, resulting in either transcriptional activation or repression (Bannister & Kouzarides., 2005). Methylation of lysine residues can be one of three forms (mono, di- or tri-methylation), whilst methylation of arginine can be either mono- or di-methylation. Methylation has been linked with both transcriptional activation and repression, depending upon which histone lysine sites are methylated (see Table 1.1) (reviewed in Kouzarides *et al*., 2007). Unlike acetylation, methylation has no impact on the net electrostatic charge between histone and DNA, instead its affects upon transcription are carried out by methylation providing a binding surface for effector proteins to bind to and perform defined tasks. For example, methylation of H3K9 acts as a binding site for the corepressor

heterochromatin protein-1 (HP1), which induces condensation of the chromatin structure, into a repressive heterochromatin form (Bannister *et al*., 2001).

Methylation can be reversed by demethylase enzymes, with LSD1 being the first demethylase discovered (Shi *et al*., 2004). LSD1 can lead to transcriptional repression and activation, depending on which lysine the methyl group is removed. Removal of the methyl group from H3K4 has been shown to repress transcription (Shi *et al*., 2004), whilst demethylation of H3K9, in conjunction with the androgen receptor, leads to transcriptional activation (Metzger *et al*., 2005). Since then, a number of other demethylases have been discovered (reviewed in Kouzarides., 2007).

Throughout this thesis, two TF families will be investigated, with regards to the regulation of pro-convulsant tachykinins and in response to ACD treatment. These being the NRSF and USF families, both of which have known roles in chromatin remodelling, and which form associations with many of the enzymes introduced here.

1.4 Neuron Restrictive Silencer Factor (NRSF)

NRSF (also termed REST), was discovered by two groups simultaneously in 1995, as a protein which binds to the NRSE (also known as repressor element-1, RE1), in the rodent SCN2a (Chong *et al*., 1995) and SCG10 genes (Schoenherr & Anderson., 1995). As the NRSE consensus sequence was found in multiple neuron-specific genes (including the M4 muscarinic acetylcholine receptor, CHRM4 (Wood *et al*., 1996); BDNF (Timmusk *et al*., 1999); Synaptosomal associated protein, Snap25 (Bruce *et al*., 2004) and L1 cell adhesion molecule, L1CAM (Kallunki *et al*., 1997)), NRSF was initially thought to function as a silencer of neuronal genes in non-neuronal cells, and hence play a key role in neuronal differentiation. Since those early findings, NRSF has been shown to play a number of roles in gene regulation and chromatin remodelling, and is implicated in a range of disorders including cancer, HD and epilepsy.

NRSF binds to the canonical 21bp NRSE site through eight C_2H_2 zinc fingers, with the consensus sequence being T(C/T)AG(A/C)(A/G)CCNN(A/G)G(A/C)(G/C)AG (Bruce *et al*., 2004; Wu and Xie., 2006) (Figure 1.6a). The use of genome wide bioinformatic assays and chromatin immunoprecipitation (ChIP) sequencing assays (ChIP-seq) have revealed a vast number of NRSEs throughout the genome, with estimates at 1892 in humans (Bruce *et al*., 2004). This has since been supported by the 1946 *in vivo* NRSF binding sites observed in human (Johnson *et al*., 2007) and 893 SACO (serial analysis of chromatin occupancy) identified NRSEs in a murine kidney cell line (Otto *et al*., 2007). The majority of these putative NRSEs are located within intergenic regions (40%), with 24% located within introns and 15% within promoter

regions (5kb upstream of TSS), consistent with other TFs, including CTCF and STAT1 (Jothi *et al*., 2008).

These genome wide screenings have revealed which nucleotides of the NRSE are important for protein-DNA binding, with nucleotides 7-9 (ACC) and 12-17 (GGACAG), shown to be critical for DNA binding, and residues 1-6 found to contribute to binding stability (Jothi *et al*., 2008). Over the past few years, the 21bp canonical sequence has been shown to be partitioned into two non-canonical half sequences, denoted the left half-site and the right half-site (Figure 1.6b) (Patel *et al*., 2007; Johnson *et al*., 2007; Jothi *et al*., 2008; Valouev *et al*., 2008). Interestingly, a recent study has revealed that neither the left nor the right half-site alone is an effective repressor, suggesting that whilst NRSF can bind to either half-site, half-site binding is not as strong or as functional as the canonical full site (Patel *et al*., 2007).

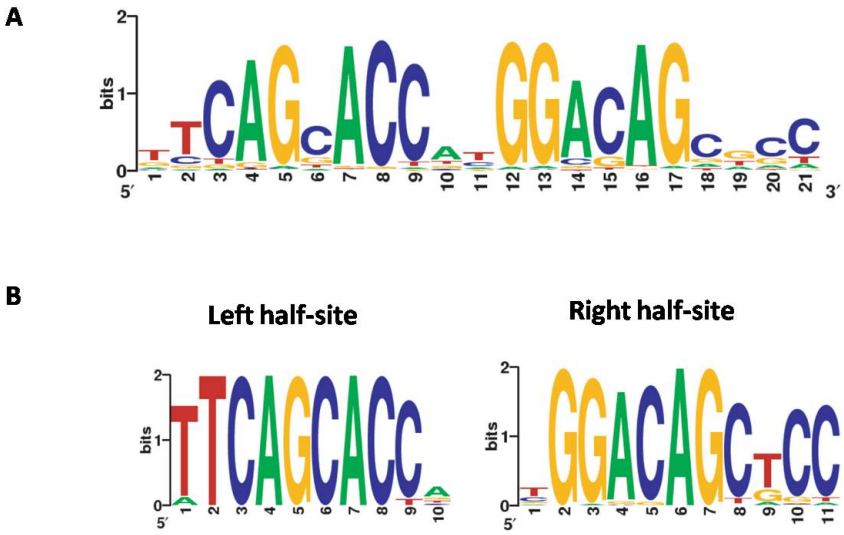

Figure 1.6 The NRSF consensus sequence (NRSE) (A) the canonical 21bp sequence of the NRSE, and **(B)** the partitioned NRSE, split into two distinct halves, the left-half site and the right-half site. (Modified from Jothi *et al*., 2008).

1.4.1 NRSF mediated gene regulation and chromatin remodelling

NRSF mediated gene regulation is coordinated through the recruitment of a battery of co-repressors and chromatin remodelling enzymes, at its two distinct domains; the N- and the C-terminal domains. At the N-terminal domain, mSin3a binds to NRSF (Grimes *et al*., 2000) and recruits two class I HDACs (HDAC1 and HDAC2) (Huang *et al*., 1999). In addition, the class II HDACs HDAC4 and HDAC5, have also been shown to be recruited by the mSin3 complex, but this association is lost following HDAC phosphorylation by calcium/calmodulin-dependent protein kinase (CAMK) (Nakagawa *et al*., 2006). The C-terminal domain recruits CoREST (Andres *et al*., 1999), with CoREST able to recruit HDAC1 and HDAC2 (You *et al*., 2001). In addition, CoREST recruits the histone H3K4 demethylase LSD1 (Shi *et al*., 2004), BRG1 (a member of the SWI/SNF chromatin remodelling family) (Battaglioli *et al*., 2002; Ooi *et al*., 2006), G9a (Roopra *et al*., 2004), the NADH-binding corepressor CtBP (Garriga-Canut *et al*., 2006) and a methyl-CpG-binding protein, MeCP2 (Lunyak *et al* 2002; Ballas *et al*., 2005) (summarised in Figure 1.7).

The formation of these complexes has a profound effect upon transcription. In the first instance, BRG1 facilitates and stabilises NRSF binding to the target NRSEs *in vivo* (Ooi *et al*., 2006). This function has been shown to require both the ATP-dependent chromatin remodelling activity of BRG1 (Ooi *et al*., 2006) and the bromodomain, which binds to acetylated H3K8 (Agalioti *et al*., 2002). It is thought that BRG1 induces chromatin remodelling to enable NRSF greater access to its target sequences. This SWI/SNF factor is shown to be critical in NRSF-mediated gene silencing, with knock-

down of BRG1 shown to induce derepression of NRSF target genes SCG10 and Synaptophysin (Watanabe *et al*., 2006).

Following NRSF recruitment to target NRSEs, the co-repressors can then start to invoke distinct changes. HDACs, as previously mentioned, remove acetyl groups from histones, resulting in a more condensed chromatin environment. Both the N- and C-terminal domains have been shown to interact with HDAC1 and HDAC2, with either domains capable of deacetylating histone 3 (H3K9 and H3K14) (Bingham *et al*., 2006). Following deacetylation of histone 3, demethylation of H3K4 (a marker of activation) can occur, mediated by the histone demethylase LSD1 (Lee *et al*., 2005). Furthermore, H3K9, now free from an acetyl group, can be dimethylated by G9a (Roopra *et al*., 2004) becoming H3K9me2, a marker for repression (Li *et al*., 2007). This process has recently been shown to be orchestrated by Mediator, which as mentioned previously, is important in transducing regulatory signals to the basal transcription machinery. Ding *et al*, found that the MED12/ MED19/MED26 interface of Mediator, is key in linking NRSF with G9a, and thus plays a role in G9a-mediated H3K9 dimethylation (Ding *et al*., 2008; Ding *et al*., 2009). Intriguingly, a second group has shown that CDYL (chromodomain on Y-like), physically bridges NRSF and G9a, with disruption of this leading to oncogenic transformation (Mulligan *et al*., 2008). The process of dimethylation of H3K9, induces the subsequent recruitment of the HP1 (Belyaev *et al*., 2004; Roopra *et al*., 2004), which in turn mediates chromatin condensation. Overall, the process of deacetylation, demethylation and methylation generates a condensed heterochromatin environment, which suppresses transcription.

In addition to chromatin remodelling, NRSF has been shown to affect transcription more directly, affecting the basal transcription machinery. NRSF has been shown to bind to TBP, in turn inhibiting PIC formation (Murai *et al*., 2004). Furthermore, NRSF has been shown to interact with Pol-II bound small CTD phosphatases (SCPs), inhibiting the activity of Pol-II (Yeo *et al*., 2005). Thus NRSF has a profound impact upon gene regulation and transcription.

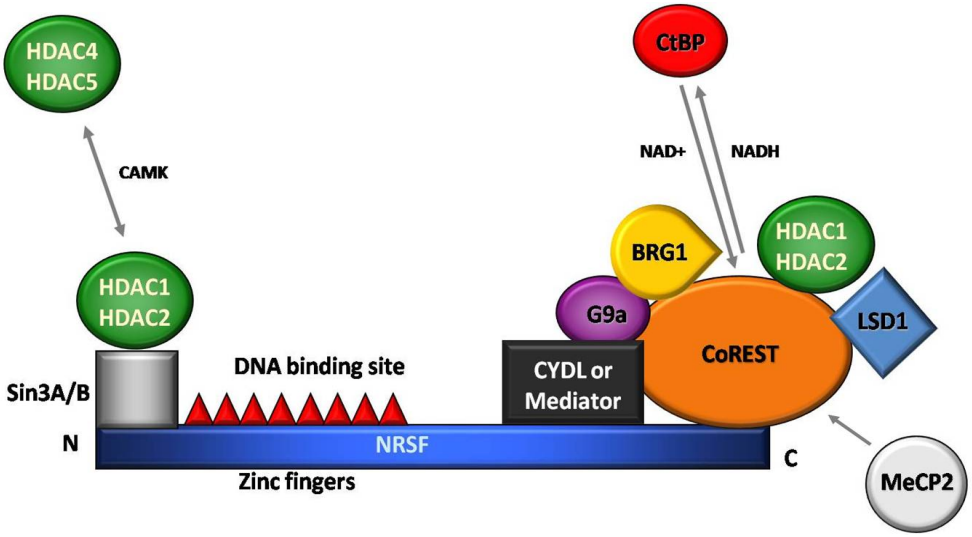

Figure 1.7 NRSF and its cofactors. Schematic representation of the cofactors which form associations with NRSF. At the N-terminal domain, the mSin3 complex forms, which recruits HDACs. The C-terminal domain, recruits a multitude of cofactors, including CoREST, HDAC1, HDAC2, LSD1, G9a and BRG. Addition recruited cofactors are also indicated including MeCP2 and CtBP.

1.4.2 Modulation of NRSF activity

NRSF is known to be expressed at a high level in non-neuronal cells, but at a much lower level in neuronal tissue. This differential expression requires specific control of NRSF regulation, and yet, despite extensive studies into NRSF-mediated gene regulation, there is little known regarding the regulation of NRSF expression itself. Both human and mouse NRSF genes contain three non-coding exons, in the 5'UTR, and three

coding regions. To date, studies have focused on the three non-coding exons (exons I, II and III). NRSF transcripts arise from one of the three non-coding exons, with the majority (80%) arising from exon I (Palm *et al*., 1998). It has been suggested that NRSF contains two (Kojima *et al*., 2001) or three (Koenigsberger *et al*., 2000) promoters, with none of these promoters exhibiting cell-specific properties. Therefore it is possible that the levels of endogenous NRSF are not fully governed by transcription *per se*, but perhaps are modulated at a post-transcriptional level (such as splicing, mRNA stability or post-translational modifications), in a cell-specific manner.

One potential candidate TF for NRSF regulation is Sp1, based upon the presence of multiple GC boxes flanking exons I, II and III (Koenigsberger *et al*., 2000). However, the effect of over-expression or knock-down of Sp1 upon NRSF expression has yet to be clarified. In addition, Nichihara *et al*, have shown that NRSF expression is mediated by the Wnt-signalling pathway, with over-expression of Wnt1 or β-catenin resulting in enhanced NRSF expression in chick embryos (Nishihara *et al*., 2003). More recently, a role for both Oct4 and Nanog TFs in regulating NRSF expression have been proposed, with Oct4 and Nanog binding sites found 2kb upstream of NRSF exon I, and knockdown of either Oct4 or Nanog found to induce a reduction in NRSF expression in mouse embryonic stem cells (ESCs) (Loh *et al*,. 2006). Finally, a role in the repression of NRSF expression has been postulated for MeCP2. MeCP2 deficiency in the brain has been shown to reduce expression of the NRSF-regulated gene BDNF, and this is thought to be due to enhanced NRSF expression. In support of this, Abuhatzira *et al*, have shown that MeCP2 binds to the NRSF promoter, and that MeCP2 knockdown induces elevated NRSF expression in human and mouse brains (Abuhatzire *et al*., 2008).

The modulation of endogenous NRSF protein levels is also an important factor which may affect the NRSF regulatory system. Recent reports have uncovered the process by which NRSF protein levels are maintained. Two groups have shown that NRSF is degraded by the ubiquitin system, with the E3 ligase responsible for orchestrating this degradation found to be the Skp1-Cul1-F-box protein complex containing the F-box protein β-TRCP (SCF $^{\beta\text{-TRCP}}$) (Westbrook *et al*., 2008; Guardavaccaro *et al*., 2008).

1.4.3 Truncated isoform sNRSF

Truncated isoforms of NRSF have been observed in both rat (Palm *et al*., 1998) and human tissue (Palm *et al*., 1999; Coulson *et al*., 2000), and have been implicated in both SE (Spencer *et al*., 2006) and neuroendocrine cancers, such as small cell lung cancer (SCLC) (Coulson *et al*., 2000). Palm *et al*, first revealed the presence of alternatively splice isoforms of NRSF in rats, with five isoforms discovered, denoted rREST1, rREST2, rREST3, rREST4 and rREST5 (Palm *et al*., 1998). I will continue to use these names for now, to distinguish between the rodent and human isoforms reported. rREST1-3 were found to arise from the addition of short sequences in rat exon IV (rREST1) and exon V (rREST2) and as a result of the loss of 8bp from exon VI (rREST3). Two other isoforms were discovered, which arise from the presence of an extra exon, designated exon N, between exons V and VI. This exon N, can be either 16bp long or 28bp long, both encoding a premature stop codon, which results in the formation of the truncated isoforms rREST4 and rREST5 respectively. These latter two isoforms lack the entire C-terminal domain, have only 5 zinc fingers, and are shown to be less repressive that wild type rREST (Palm *et al*., 1998). The abundance of these

isoforms is relatively low in brain tissue, with rREST4 accounting for only 1% of the total rREST transcripts (Palm *et al*., 1998).

Palm *et al*, later discovered that rREST4 and rREST5 were conserved in mice, with two truncated isoforms isolated, arising from either a 16bp or 28bp exon N, matching that found in the rat. In humans, the presence of an extra exon (N) between exons V and VI, was also observed, which could be either a mere 4bp or 62bp in length. The 62bp insertion was found to have almost perfect homology to the rat and mouse exon N sequence, at its 3' end, except for a single nucleotide difference (Palm *et al*., 1999). Like the rodent isoforms, this 62bp insertion encodes for a premature stop codon, resulting in a truncated isoform. Since these findings, our group has also published on the presence of a truncated isoform of NRSF in human SCLC cells (Coulson *et al*., 2000). Our group discovered a 50bp sequence insertion between exons V and VI, again designated exon N, encoding a premature stop codon, thus mirroring in part, that found in the rodent. The resulting truncated isoform is designated short NRSF or sNRSF. As with the rodent truncated isoforms rREST4 and rREST5, sNRSF has only five zinc fingers, and is missing the entire C-terminal domain (Coulson *et al*., 2000). As mentioned previously, the 3' end of the 62bp sequence identified by Palm *et al*, had an almost perfect homology to the rodent exon N sequence, and the latter 50bp sequence, reported by Coulson *et al*, is contained within this 62bp sequence. The addition 12bp found by Palm *et al*, are a perfect match for the extra 12bp found in the rodent, which distinguishes between rREST4 and rREST5. This suggests that two truncated isoforms may occur in human tissue.

Whilst the role of full-length NRSF is well established, a role for the truncated isoform is poorly characterised. Based on the absence of the C-terminal domain, one may postulate that sNRSF may function differently to NRSF, and it has been suggested that the truncated isoform may act as an activator rather than a repressor (Lee *et al*., 2000). The early findings that NRSF isoforms are up-regulated following KA treatment suggest a possible role in seizure (Palm *et al*., 1998), which was later confirmed by our group (Spencer *et al*., 2006). The rodent rREST4 was found to be up-regulated following 3hrs KA, which correlated with an increase in TAC1 mRNA expression, supporting the postulated role in transcriptional activation for the truncated isoform (Spencer *et al*., 2006). Various other studies have suggested that truncated NRSF may exert a different regulatory role over NRSF targets, compared to full-length NRSF. For example, Magin *et al*, revealed that whilst NRSF represses the human synapsin I promoter, the truncated NRSF does not (Magin *et al*., 2002). Alternatively, the truncated isoform has been proposed to act as an antagonist of NRSF-mediated gene repression. NRSF repression of both the BDNF promoter and the cholinergic gene locus has been shown to be alleviated by the truncated isoform (Tabuchi *et al*., 2002; Shimojo & Hersh., 2004). In fact, rREST4 has been shown to inhibit NRSF binding to the cholinergic gene NRSE, leading to de-repression and activation of the cholinergic gene locus (Shimojo *et al*., 1999). Furthermore, whilst full-length NRSF has been shown to almost completely inhibit the induction of gene transcription following glucocorticoid stimulation in non-neuronal cells, the truncated isoform, in contrast, was found to stimulate gene transcription, shown to be mediated by the chromatin remodelling factor

hBrm (Abramovitz *et al*., 2007). Thus, the NRSF regulatory system may be impaired in conditions or disorders with elevated truncated isoform expression, such as SE or SCLC.

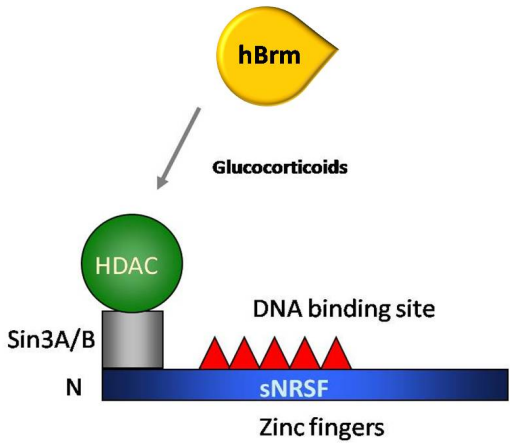

Figure 1.8 The human truncated isoform sNRSF. The human truncated isoform of NRSF, sNRSF, consists of the N-terminus of full-length NRSF, and five zinc fingers. The chromatin remodelling complex hBrn has been shown to interact with this isoform in response to glucorticoid stimulation.

1.4.4 NRSF Localisation

The localisation of any protein is an important factor in determining its function, and this is particularly true when considering TFs which require nuclear localisation to function as regulators of gene expression. NRSF has been shown to exhibit a cytoplasmic localisation in untreated neurons (Zuccato *et al*., 2003; Spencer *et al*., 2006), and is known to translocate into the nucleus following KA-induced SSSE treatment in rat hippocampi (Spencer *et al*., 2006). In contrast, the truncated isoform has been shown to be localised in the nucleus in rat (Spencer *et al*., 2006), mouse (Lee *et al*., 2000b) and human (Magin *et al*., 2002), and was found to translocate in the opposite direction, into the cytoplasm, in KA-treated rat hippocampi (Spencer *et al*., 2006). Thus understanding the mechanisms which govern NRSF isoform localisation is an important step in elucidating their function. Two distinct proteins have been found to be important

in regulating the nuclear translocation of NRSF, these being Huntingtin (Zuccato *et al*., 2003) and the REST/NRSF interacting Lin-11, Isl-1, Mec-3 (LIM) domain protein (or REST interacting LIM protein, denoted RILP) (Shimojo & Hersh, 2003).

Huntingtin has been shown to interact with NRSF and sequester NRSF in the cytoplasm (Zuccato *et al*., 2003), whilst RILP has been shown to interact with NRSF, particularly the N-terminal domain common to both NRSF and sNRSF, and is thought to act as a nuclear receptor for these TFs. In support of this, both NRSF isoforms were found to traffic from the nucleus into the cytoplasm, following RILP knock down (Shimojo & Hersh, 2003). More recently, Shimojo has shown that the nuclear translocation of NRSF is modulated by a complex containing both Huntingtin and RILP, and a third 'gluing' protein, known as Dynactin p150^{GLUED}, which brings together Huntingtin, RILP and NRSF (Shimojo., 2008). In this complex, only RILP and Dynactin p150^{GLUED} interact with NRSF, whilst Huntingtin's association with NRSF is indirect. Finally, a fourth protein, HAP-1 (Huntingtin-associated protein 1) was also found to also bind to this complex, but this interaction was found to prevent nuclear translocation, and is thus important in cytoplasmic sequestering of NRSF (Figure 1.9) (Shimojo., 2008).

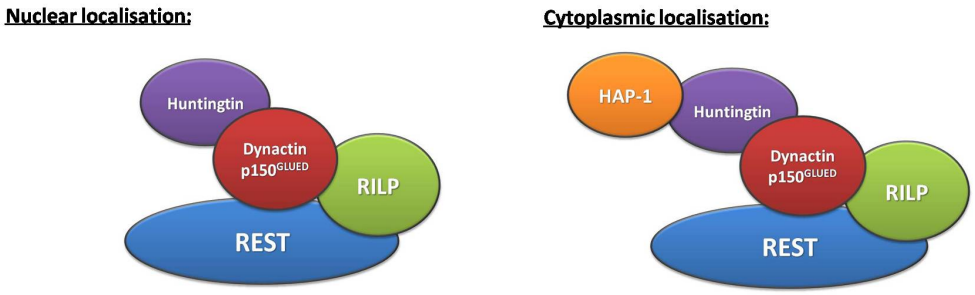

Figure 1.9. Cellular localisation of NRSF is modulated through a multi-protein complex incorporating RILP, Dynactin p150^{GLUED} and huntingtin, resulting in NRSF locating within the nucleus. HAP-1 prevents this complex entering the nucleus, and thus sequesters REST in the cytoplasm.

1.4.5 NRSF & Disease

As has been previously discussed, NRSF has the capability of regulating transcription of a multitude of genes, and as such, it is perhaps unsurprising that aberrant NRSF expression has been correlated to a host of disorders including HD (Zuccato *et al*., 2003; Zuccato *et al*., 2007), epilepsy (Palm *et al*., 1998; Spencer *et al*., 2006; Bassuk *et al*., 2008), Down's syndrome (Bahn *et al*., 2002; Canzonetta *et al*., 2008), X-linked mental retardation (Tahiliani *et al*., 2007), cognitive disfunction (Miyajima *et al*., 2009) and cancer.

1.4.5.1 Cancer

NRSF has been implicated in a range of cancers which includes neuroblastomas (Palm *et al*., 1999; Lawinger *et al*., 2000; Fuller *et al*., 2005), SCLC (Coulson *et al*., 2000) and colorectal cancers (Westbrook *et al*., 2005). Interestingly NRSF has been shown to have both tumour suppressor and oncogenic functions. NRSF knockdown has been shown to induce oncogenic transformation of immortalised primary human cells, via the de-repression of the proto-oncogene TrkC (Mulligan *et al*., 2008), indicating a potential tumour suppressor role. NRSF has also been identified as a tumour suppressor in epithelial tissue, with NRSF expression shown to be down-regulated due to an increase in SCF $^{\beta\text{-TRCP}}$, resulting in oncogenic transformation (Westbrook *et al*., 2008). Furthermore, NRSF is proposed to have tumour suppressor properties with regards to breast cancer, through the repression of TAC1 (Greco *et al*., 2007). In support of this, NRSF expression is shown to be inversely proportional to tumour cell aggression (Reddy *et al*., 2009).

Conversely, NRSF has also been implicated as an oncogenic factor. NRSF has been shown to be markedly up-regulated in many medulloblastomas (Lawinger *et al*., 2000; Watanabe *et al* 2004; Su *et al*., 2004), correlating with the observed repression of many NRSF-regulated genes, such as synapsin. This suggests that it is the NRSF repressor function that is important in tumourigenesis. In support of this, REST-VP16 over-expression induces activation of NRSF-target gene expression and blocks of tumour generation (Lawinger *et al*., 2000). Abnormal expression of NRSF, in conjunction with Myc, in NS cells and neuronal progenitor cells, has also been shown to induce the formation of cerebellum-specific tumours, via the blockade of neuronal differentiation (Su *et al*., 2006), thus highlighting NRSFs propensity to be tumourigenic.

1.4.5.2 Huntington's Disease (HD)

HD is an incurable inherited disease, caused by a CAG-expansion in the gene encoding the huntingtin protein, resulting in a mutant protein. This disorder causes severe dysfunction of motor and cognitive processes, resulting from neuronal cell death. The length of the CAG-expansion has been directly correlated with the severity of the disease. NRSF has been implicated in HD due to the role of huntingtin in determining NRSF localisation, with huntingtin important in governing the cytoplasmic localisation of NRSF. The mutant huntingtin protein is unable to bind to NRSF due to the CAG-expansion, resulting in its failure to sequester NRSF in the cytoplasm, leading to enhanced nuclear NRSF accumulation (Zuccato *et al*., 2003). This leads to elevated suppression of the neuronal survival factor, BDNF, contributing to neuronal death. NRSF therefore has an important role to play in HD, and Zuccato *et al*, have recently shown that NRSF binding to target gene NRSEs (including BDNF, Syn1, Chrm4, Drd3,

Penk1, Chrnb2) is up-regulated in a HD cell line model, resulting in an corresponding suppression of these genes (Zuccato *et al*., 2007).

1.4.5.3 Epilepsy

As mentioned at the beginning of this thesis, epilepsy is a commonly acquired, chronic neurological disorder, affecting more than 50 million people worldwide. The principle hypothesis of this thesis is that epilepsy arises following genome wide changes in chromatin structure and in turn gene expression profiles, following an initial insult, which increases the propensity of having future, spontaneous seizures. My hypothesis is that NRSF is a major factor in governing such changes, and throughout this thesis I aim to explore the roles of NRSF in relation to epilepsy, particularly its role in regulating pro-convulsant neuropeptide gene expression. The importance of NRSF in epilepsy has come to fruition in the past decade. In the late 1990's, Palm *et al* revealed that NRSF and its truncated isoform, are markedly up-regulated following treatment with the chemo-convulsant KA (Palm *et al*., 1998). This has since been confirmed by our own group, with NRSF isoforms found to be dynamically up-regulated during both PPS and KA SSSE models (Spencer *et al*., 2006). Following these findings, a number of genes found to be of importance in seizure, including TAC1 and BDNF, have been shown to be regulated by NRSF (Quinn *et al*., 2002; Spencer *et al*., 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009; Timmusk *et al*., 1999; Zuccato *et al*., 2003). These findings suggested a role of NRSF in governing gene expression in response to a seizure. Consistent with this, are the findings that 2-Deoxy-D-glucose (2DG) reduces the progression of epilepsy, through a NRSF-CtBP-dependent manner. The ketogenic diet is an anti-epileptic diet used to treat drug-resistant TLE. Carriga-Canut *et al*,

discovered that inhibition of glycolysis (the hallmark affect of the ketogenic diet), enables the NAD-sensitive compressor CtBP, to join NRSF at target NRSEs, inhibiting seizure-induced increases in BDNF and TrkB expression *in vivo* (Carriga-Canut *et al*. ,2006). Thus suggesting that NRSF mediating gene regulation is a potentially a pro-convulsant mechanism, causing abnormal gene expression.

In addition to changes in expression, NRSF has been shown to translocate into the nucleus following KA-induced SSSE in rodents (Spencer *et al*., 2006), which may lead to aberrant gene expression, akin to that observed in HD. The importance of NRSF localisation in epilepsy has recently been substantiated, with a homozygous mutation in the human RILP gene having been shown to cause an autosomal-recessive progressive myoclonus epilepsy (PME)-ataxia syndrome (Bassuk *et al*., 2008). This mutation is located within the RILP protein binding domain, and was found to disrupt the RILP-NRSF interaction, resulting in enhanced nuclear localisation of NRSF. This is consistent with the translocation of NRSF observed following KA treatment in rats (Spencer *et al*., 2006), suggesting that NRSF localisation has an important role to play in SSSE. Thus there are multiple indicators implying that NRSF is a key factor in the progression of epilepsy, and this is an issue which will be explored further throughout this thesis.

1.5 Upstream Stimulatory factors (USFs)

A second class of TFs will be explored throughout this thesis, these being the upstream stimulatory factors USF1 and USF2. The USF genes are members of the eukaryotic evolutionary conserved bHLH leucine zipper TF family. They recognize and bind to the canonical E-box regulatory element (CANNTG, whereby N denotes any nucleotide) with high affinity, with E box motifs often located within 500bp of a target gene's TSS (Rada-Iglesias *et al*, 2008). Whilst an association between the USF TFs and epilepsy has yet to be established, there are a number of reports that suggest USF may play a role in this disorder. Firstly, Sirito *et al*, observed that USF knockout mice were more prone to spontaneous seizures compared to wild types (Sirito *et al*., 1998), and later reports revealed that related bHLH factors Mash1, Id2 and Hes5 are modulated in *in vivo* rodent seizure models (Elliot *et al*., 2001). In addition, the USF TFs have been shown to regulate a host of neuronal genes, implicated in epilepsy including BDNF (Tabbuchi *et al*., 2002b), the $GABA_B$ receptor (Steiger *et al.,* 2004) and the potassium-chloride co-transporter KCC2 (Markkanen *et al*., 2008). Furthermore, our lab has previously shown a role for USF TFs in the regulation of the pro-convulsant SP-encoding gene TAC1 (Paterson *et al*., 1995; Gerrard *et al*., 2005). Consequently a putative role for such TFs in mediating the progression of epilepsy can be proposed, and shall be explored in this thesis, in a manner similar to that for NRSF.

The USF proteins were first identified in human cells due to their involvement in the transcriptional regulation of the major late adenovius promoter (Ad2MLP) (Carthew *et al*, 1985; Sawadogo & Roeder, 1985). USF was found to elevate promoter activity 10-20 fold, through binding to a -63 to -53 E-box site (similar to that in TAC1), suggesting

a potential role in transcriptional activation. The purification of USF from HeLa nuclei presented two similar proteins of 43 and 44kDa in size (Sawadago *et al,* 1988), which were later termed USF1 and USF2, respectively (Gregor *et al*, 1990; Sirito *et al*, 1992). They are ubiquitously expressed proteins, with elevated levels observed in the brain (Sirito *et al*, 1994), and specifically in the nuclei of neurons in the hippocampus, a critical brain region in epilepsy (Wasterlain *et al*., 1996). A role for USF1 and USF2 in neuronal development and embryogenesis has also been suggested, following the observation that USF1/USF2 compound mutant mice suffered embryonic lethality (Sirito *et al*, 1998). This has been recently supported following the observation of enhanced levels of USF1 and USF2 within the neural tissue and neural crest, during the progression into the neurula stage in *Xenopus larvis* development (Fujimi *et al,* 2008).

USFs are known to form dimers via their HLH-leucine zipper domains, with heterodimers (USF1/USF2) more common than the respective homodimers (Sirtio *et al*, 1992). Despite having similar DNA binding properties, USF dimers may modulate target gene expression, differentially, via the establishment of specific interactions with different TFs (Kirschbaum *et al*, 1992). For example, USF1 has been shown to interact with the Cha bHLH TF *in vitro* and *in vivo*, resulting in the inhibition of USF1 activity (Rodriguez *et al*, 2003). Furthermore, interactions between USF1 and general and cell-specific TFs SP1, Pea3 and MTF1, respectively, has been shown to lead to cooperative transcriptional regulation of target genes (Andrews *et al*, 2001).

As I have previously hypothesised, the progression of epilepsy, from an initial insult to spontaneous seizures, may arise due to global, long-term changes in gene expression patterns, perhaps via chromatin remodelling. USFs have been implicated in

chromatin remodelling through the recruitment of enzymes involved in acetylation and methylation of histones, and thus may play a role in such remodelling in response to a seizure. USF1 has been shown to mediate recruitment of PCAF, which acetylates histones H3 and H4 (Schiltz *et al*, 1999) and SET7/92 that methylates histone H3K4 (West *et al*, 2004) a marker of active chromatin. Furthermore, it has been shown that regions enriched by USF1 or USF2 binding, are also significantly enriched with histone H3 acetylation (Rada-Iglesias *et al*, 2008), and that USF1 interacts preferentially with highly acetylated H4 histones (Vettese-Dadey *et al*, 1996). Taken together, USFs exhibit the capacity to mediate a change in the chromatin environment, from a repressive heterochromatin formation into the more open and active euchromatin environment, and hence promoting gene expression. The regulation of gene promoters by USF is thus a complex and dynamic process, incorporating multiple TFs, together with the recruitment of chromatin remodelling enzymes, enabling chromatin reorganisation, in turn modulating transcriptional regulation (Sha *et al*, 1995).

1.5.1 The USF TFs in disease

The USF TFs have been shown to play important roles throughout the body, including mediating the immune response to factors such as viral invasion, and the skin's melanin-production response to UV-radiation (reviewed in Corre & Galibert., 2005). In addition USF has been shown to have a key function in coordinating cellular differentiation and proliferation, and subsequently is important in disorders characterised by aberrant cellular proliferation, i.e. cancers. More specifically, USF has been shown to have anti-proliferative roles, with USF shown to inhibit both c-Myc and Ras-induced transformation of primary rat cells (Aperlo *et al*., 1996; Luo & Sawadogo.,

1996), and inhibits cellular proliferation in thyroid follicular cells (Jung *et al*., 2007). Consistent with a role in cellular proliferation, a number of USF-regulated genes have established roles in differentiation and proliferation, such as human telomerase reverse transcriptase (hTERT) (Goueli & Janknecht., 2003). This particular gene is of interest as hTERT expression is known to be up-regulated in more than 85% of tumour cells. USF has been shown to mediate hTERT promoter activity, with both USF1 and USF2 shown to inhibit hTERT promoter driven luciferase reporter gene activity, suggesting that USF TFs repress hTERT expression. Consistent with this, USF1 and USF2 expression was found to be repressed in tumour tissue, correlating with a marked increase in hTERT expression (Chang *et al*., 2005). This suppression was found to be controlled via direct binding of USF proteins to an E box located within the hTERT promoter.

Given the importance of its role in the regulation of hTERT and in governing cellular proliferation, USF has also been shown to regulate the expression of a number of tumour suppressor genes, including p53 and APC (Jaiswal & Narayan., 2001; Reisman & Rotter., 1993). Thus a role in tumour suppression can be attributed to the USF TFs, which is consistent with the observed reduction in USF expression in tumour tissues (Chang *et al*., 2005). More recently, Chen *et al*, have shown a tumour suppressor role for USF2 in prostate cancer, with the over-expression of USF2 in three prostate cancer cell lines, causing a substantial inhibition in both anchorage-independent growth and invasion capability of these cells (Chen *et al*., 2006). Finally, the USF TFs have been shown to play an important role in lung cancers, particularly SCLCs (Coulson *et al*., 2003; Ocejo-Garcia *et al*., 2005), in which NRSF has also been demonstrated to play an important role (Coulson *et al*., 2000).

1.6 Anti-convulsant drugs (ACDs)

The primary intention of ACD treatment is to prevent epileptic seizures. ACDs exert their anti-convulsant activity through single or multiple targets, which include ion channels, neurotransmitter receptors and neurotransmitter metabolic enzymes (Rogawsi & Loscher., 2004a). The ultimate aim is to modulate neuronal firing and to inhibit the spread of abnormal firing to distant parts of the brain. Unfortunately, despite ongoing research and the emergence of new generation ACDs, approximately 50% of patients treated with modern ACDs continue to experience seizures (Pitkanen,, 2002). In addition to their roles in protection against seizure, ACDs have been shown to be beneficial in the treatment of migraine, neuropathetic pain, bipolar disorder (BD), anxiety, schizophrenia and alcohol dependence and withdrawal (Rogawsi & Loscher., 2004b).

To date, there are over 20 ACDs approved for use in the UK (British National Formulary, No.55, March 2008), each of which can be separated into groups based upon their mechanisms of action and which type of epilepsy the drug can be used for. For example, carbamazepine (CBZ), phenytoin (PHY) and lamotrigine (LMT) all are proposed to function by targeting voltage-gated sodium channels, such as the type II voltage-gated sodium channel SCN2a, and all three are approved for use in treating GTC and Partial seizures, based on this function (Rogawsi & Loscher., 2004a). These drugs have been shown to block high-frequency repetitive spike firing, which is hypothesised to occur during the spread of seizure activity, without affecting normal neuronal firing (Xie *et al.,* 1995). Other drugs, such as Vigabatrin, work by enhancing synaptic inhibition by targeting $GABA_A$ receptors, enhancing inhibitory GABAergic

signalling (Hanaya *et al*., 2002). Alternatively, excitatory neurotransmitter systems can be impaired by blocking glutamate receptors NMDA or AMPA using Felamate or topiramate respectively (Yang *et al*., 2007; Gryder & Rogawski., 2003).

1.6.1 Voltage-Gated Sodium Channels

Sodium channels are important in the rising phase of neuronal action potentials. Following depolarisation, sodium channels undergo conformational changes, converting the channel from its resting 'closed' non-conductive state, to an 'open' conformation which enables Na^+ flux. The channel is then rapidly repolarised, returning to its 'closed' state. ACDs such as CBZ, PHY and LMT bind only weakly to target sodium channels (e.g. SCN2a) at hyper-polarised membrane potentials, but are able to bind strongly following depolarisation, markedly increasing their inhibitory properties, (Willow el al., 1985; Xie *et al*., 1995; Remy el al., 2003). Interestingly, blockade of sodium channels by PHY and LMT has a slow onset, and when the drugs are bound, the interaction is tight and slow to unbind. An advantage of this slow onset is that it enables normal neuronal firing to continue. Furthermore, as binding is slow, and depolarisation and repolarisation is rapid, then binding of PHY and LMT can only bind if depolarisation is prolonged, which is characteristic of certain types of epilepsy, such as focal epilepsy.

It has been shown that despite structural dissimilarities (see figure 1.10), CBZ, PHY and LMT all bind to a common recognition site on the inner pore of the SCN2a sodium channel (Kuo *et al*., 1998). Each drug contains a motif of two phenyl groups, which are proposed as being crucial to drug binding to the S5 and S6 transmembrane domain units. Furthermore, specific residues phenylalanine (F1764) and tyrosine

(Y1771) in the S6 domain have been shown to be important in PHY and LMT drug action (Ragsdale *et al*., 1996; Liu *et al*., 2003).

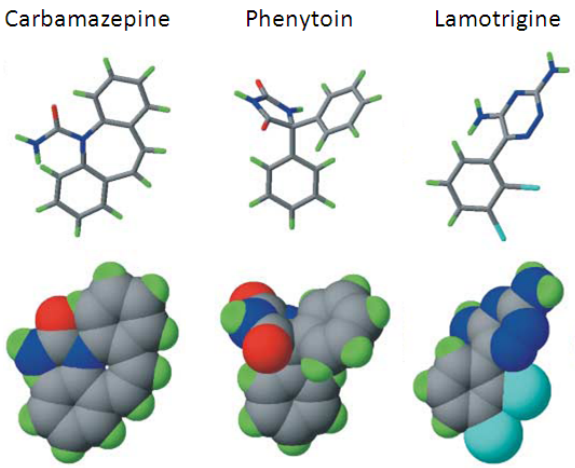

Fig 1.10 Structure of the three ACDs utilised in this thesis; CBZ, PHY and LMT. Modified from Rogawski & Loscher 2004a.

1.6.3 Carbamazepine (CBZ)

CBZ (5H-dibenz[b,f]azepine-5-carboxamide) has been one of the most commonly prescribed ACDs since it was first used for the treatment of epilepsy in the mid-1960s (Ambrosio *et al*., 2002; LaRoche and Helmers, 2004). It has also been prescribed for the treatment of neuropathic pain and approved for use in the treatment of psychiatric disorders including mania (Ettinger & Argoff, 2007). CBZ is structurally distinct from other ACDs, being an iminodibenzyl derived, tricylic compound with a short amide side chain (Ambrosio *et al*, 2002). Despite this, CBZ is thought to function by targeting the same voltage-dependent, tetredoxin-resistant sodium channels as PHY and lamotrigene (Kuo, 1998). By impairing the activity of these sodium channels, CBZ reduces the frequency of repetitive action potentials, thus lowering neuronal firing

(Elliot, 1990). CBZ also functions by affecting both glutamate and GABA transmission and regulation. Glutamate is transported by excitatory amino acid transporters (EAAT), from the extracellular to the intracellular space, preventing extracellular accumulation of glutamate, and thus preventing enhanced neuronal firing. These transporters have been implicated in epilepsy, following the discovery that the dysfunction of EAAT3 induces seizure in rodents (Sepkuty *et al*, 2002). CBZ has been shown to enhance the activity of this glutamate transporter (Lee *et al*, 2005), indicating that it can function by targeting glutamate regulatory mechanisms. Furthermore, CBZ has been found to inhibit sodium channel mediated glutamate release in a dose dependent manner (Sitges *et al*, 2007). It is thus apparent that CBZ is capable of regulating key excitatory neurotransmitter pathways.

As well as regulating excitatory neurotransmitter pathways, CBZ also affects inhibitory neurotransmitter pathways. GABA is known to be degraded by multiple enzymes, including Succinic semialdehyde dehydrogenase (SSADH). This enzyme is implicated in epilepsy, with SSADH deficient mice exhibiting enhanced seizure activity and an epileptic phenotype (Cortez *et al*, 2004; Gupta *et al*, 2004). CBZ has been shown to physically interact with SSADH and decrease its activity (Sawaya *et al*, 1975), suggesting that CBZ could enhance the half-life of GABA. Consistent with this, GABA levels were found to be elevated in the hippocampus following CBZ treatment in rats (Yoshida *et al*., 2007). Taken together, CBZ clearly regulates both excitatory and inhibitory signalling pathways, generating an inhibitory neurotransmitter environment within the brain.

1.6.4 Phenytoin (PHY)

PHY (5,5-diphenylhydantoin), like CBZ has been used in the treatment of seizure for over fifty years, since it was approved for use by the FDA in 1953. Despite sharing a common binding target with CBZ and LMT, PHY is structurally quite distinct, containing a chemical structure which is traditionally viewed as a key structure for anti-convulsant activities, known as the ureide structure (Figure 1.10). In addition to its use in the treatment of seizure, PHY has been used to treat both neuropathetic and trigeminal pain, as well as being routinely used in the treatment of the manic phase of BD (Rogawski & Loscher., 2004b).

PHY has been shown to be effective in blocking the development of SSSE, if administered prior to PPS. However, PHY is shown to be less effective when given 10mins after PPS (Chen & Wasterlain., 2006), which may be due to the slow-onset of PHY's affects. In addition to sodium channel blockade, PHY has been show to reduced spontaneous release of glutamate, whilst enhancing spontaneous GABA release in rat entorhinal cortex (Cunningham *et al*., 2000a). Furthermore, Sitges *et al* revealed that PHY, like CBZ blocks sodium-channel mediated release of glutamate in the rat hippocampus (Sitges *et al*., 2007), indicating that PHY can modulated the release of both excitatory and inhibitory neuropeptides, shifting the balance towards neuronal inhibition.

1.6.5 Lamotrigine (LMT)

LMT is a member of the 2nd generation of ACDs, which targets the same sodium channels as CBZ and PHY (Kuo*.,* 1998), but has a more simplistic structure containing

just two aromatic rings (Figure 1.10). Unlike CBZ and PHY, LMT has been shown to selectively inhibit late epileptiform after-discharges, without affecting initial action potential responses (Xie *et al*., 1995). Furthermore, LMT has also been shown to inhibit high voltage calcium channels (Stefani *et al.,* 1996; Wang *et al*., 1996), which may explain why LMT is useful for the treatment of Absence seizures and Myoclonic seizures (Posner *et al.,* 2005; Auvin., 2008), whilst PHY and CBZ are not. These high voltage calcium channels are known to be crucial for abnormal oscillatory behaviour underlying generalized absence seizures (Huguenard *et al*., 1996).

LMT has been shown to delay the development of kindling (McNamara *et al* 1995), and like PHY, LMT has also been shown to impair spontaneous glutamate release, whilst enhancing spontaneous GABA release in rat entorhinal cortex (Cunningham *et al*., 2000b). In addition, LMT (like CBZ) has been shown to up-regulate BDNF expression in rat frontal cortex (Chang *et al*., 2009), indicating that LMT promotes an inhibitory neuronal phenotype, whilst enhancing neuronal survival. Consistent with this, LMT has been shown to alleviate hippocampal damage in rats following PPS inducing SSSE (Halonen *et al*., 2001), as well as exhibiting neuroprotective properties in KA-induced SE models (Maj *et al*., 1998). Perhaps because of this, LMT has been noted to induce fewer side effects, on the basis of less patient withdrawal from treatment, compared to both CBZ (Brodie *et al.,*1995) and PHY (Steiner *et al.,* 1999).

1.7 Cocaine

Cocaine is a psychostimulant drug which exerts its affects predominantly via dopamine transporter (DAT) blockade, preventing dopamine uptake and resulting in elevating dopamine concentrations in the synaptic cleft. This causes the well-documented euphoric psychological effect. In addition, cocaine is known to inhibit other neurotransmitter transporters including SLC6A4 and norepinephrine (NET). An increasing appreciation of cocaine-induced seizures has arisen in the past 20 years, with cocaine-induced seizures documented in both humans and rodents, with short-term GTC seizures observed in humans (Lowenstein *et al*., 1987) and short seizures exhibiting clonic features observed in rodents (Hanson *et al*., 1999) (see Lason., 2001, for a review). Other reports have revealed that repeated subconvulsive doses of cocaine can induce kindling (Miller *et al*., 2000). One potential cause of this is cocaine's effect on the dopaminergic system, which has become increasingly associated with epilepsy. Hyperactivity and spontaneous seizures have been reported in mice following the loss of D1 dopamine receptor expressing cells (Gantois *et al*., 2007), whilst D2 and D4 dopamine receptor knock-out mice have been shown to be more susceptible to pharmacological induced seizures (Weinshenker & Szot., 2002). Furthermore, D3 dopamine receptor agonists have been shown to exhibit pro-convulsant properties, which can be reversed using D3 dopamine receptor antagonists (Witkin *et al*., 1998). In addition, a number of ACDs have been trialled to treat cocaine addiction, including CBZ (Brady *et al*., 2002; Lima *et al*., 2002; Campbell *et al*., 2003).

Another potential mechanism for cocaine-induced seizures could be the modulation of TFs such as NRSF, which I postulate to be an important TF in the

progression of epilepsy. Whilst the impact of cocaine upon the NRSF regulatory system has yet to be determined, NRSF has recently been shown to modulate the expression of CART. CART is a peptide shown to be up-regulated following cocaine treatment (Douglass *et al*., 1995) and is intriguingly found to be co-localised with the NRSF-regulated SP in the rodent nucleus accumbens. In addition, cocaine treatment has also been shown to up-regulate the expression of the NRSF-regulated gene, BDNF (Zhang *et al.,* 2002; Grimm *et al.,* 2003; Filip *et al*., 2006). These findings suggest that cocaine may indeed affect the NRSF regulatory system, an issue I aim to clarify in chapter 8.

Furthermore, an apparent overlap between the proconvulsant tachykinin signalling system and cocaine has become apparent in the past 5 years, with antagonism of NK1R, shown to reduce behavioural sensitization to cocaine in rats (Davidson *et al*. 2004). Similarly, NK3R has been shown to coordinate cocaine's psychostimulant affects. Notably NK3R antagonism has been found to block these psychostimulant properties in both rats and primates (Jocham *et al*., 2006), whilst the NK3R agonist, senktide, has been shown to potentiate cocaine-induced dopamine release (De Souza Silva *et al*., 2006). This overlap with the pro-convulsant signalling system may be another aspect of cocaine-induced seizures.

1.8 Aims & Objectives

The progression from an initial insult to recurrent, spontaneous seizures, or 'epilepsy', involves dramatic cellular and molecular changes, including structural modifications of neuronal circuitry and gene expression profile changes. I postulate that the latter could be governed by long-term changes in chromatin structure, resulting in aberrant gene expression, leading to a hyper-excitable neuronal phenotype. A multitude of TFs have the capability to induce such chromatin remodelling, and in this thesis I will focus on two distinct TFs families, NRSF and USF. I aim to explore their relevance to epilepsy in terms of governing gene expression, and as a response to both pro and anti-convulsant treatments.

In the first instance I aim to explore the regulatory roles of these two TFs upon two pro-convulsant neuropeptides, the tachykinin genes TAC3 and TAC1. With regards to TAC3, I aim to clone the NKB promoter region into a reporter gene construct, to test if these TFs modulate the NKB promoter activity. In addition, I aim to explore the effect of NRSF isoforms and the USF1 and USF2 on endogenous NKB expression. With regards to TAC1, I aim to further explore the already documented roles of NRSF and USF in the regulation of TAC1 (Quinn *et al.,* 2002; Spencer *et al.,* 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009; Paterson *et al*., 1995; Gerrard *et al*., 2005), by exploring a potential synergistic regulation of TAC1 by these two TFs, akin to the documented NRSF and NFκB synergy (Greco *et al*., 2007). Our theory that a relationship between NRSF and USF-mediated regulation exists, is born out of the overlapping regulation of BDNF, AVP, and TAC1 (Timmusk *et al*., 1999; Tabuchi *et al*., 2002a; Coulson *et al*., 1999; Coulson *et al*., 2003).

To gain a greater insight into the relevance of these TFs in epilepsy, I also sought to investigate how NRSF and USF respond to ACD treatment. Potential factors for ACD modulation include mRNA expression, cellular localisation and DNA binding ability. Each of these factors will be explored for the NRSF isoforms and the USF genes USF1 and USF2, in response to three ACDs (CBZ, PHY and LMT), which share a common initial target (sodium channel), but can induce different downstream modifications. Furthermore, I aim to explore global gene expression changes following the over-expression of both the full-length NRSF and a truncated version analogous to sNRSF, via microarray analysis. NRSF isoforms have been shown to be up-regulated following 3hrs (Spencer *et al*., 2006) and 4hrs (Palm *et al*., 1998) KA administration in rats, representing an initial insult. By over-expressing these isoforms and exploring global gene expression changes, it is hoped one can unravel the more long term changes in gene expression which may lead to an enhanced susceptibility to future seizures, and hence gain a greater insight into the process of epileptogenesis.

Finally, due to an increasing appreciation of the dopaminergic system in seizures and the increase in cocaine-induced seizure reports, I wish to explore the impact of cocaine treatment on both NRSF and USF. One hopes that by comparing how these TFs respond to cocaine to how they respond to both pro- and anti-convulsant chemical stimulation, underlying mechanisms or pathways which govern these TFs may be revealed, which may help in the generation of future anti-epileptic strategies.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasmids and vectors

Plasmid Name	Description	Source
Reporter Gene Constructs		
pNKB -757 +181 luc (pNKB-757)	pGL3basic luciferase reporter gene backbone containing a fragment of the human NKB proximal promoter spanning -757 to +181, cloned upstream of the luciferase reporter gene DNA.	See Chapter 3.
pNKB -289 +181 luc (pNKB-289)	pGL3basic luciferase reporter gene backbone containing a fragment of the human NKB proximal promoter spanning -289 to +181, cloned upstream of the luciferase reporter gene DNA.	See Chapter 3.
rTAC1 -865 +92 luc (-865 TAC1)	Recombinant adeno-associated virus 2 (rAAV2) plasmid backbone, containing a fragment of the rat TAC1 proximal promoter spanning -865 to +92 and the luciferase reporter gene DNA in place of the AAV genome.	Harrison *et al*., 1999
rPPT-A -865 +92 luc (-60 TAC1)	As the rTAC1 -865 +92 luc but with a 10bp insertion at -60 position in the rat TAC1 promoter disrupting the -60 E Box motif.	Paterson *et al*., 1995
NRSF expression constructs		
REEX1	pCMV expression vector containing the full length human NRSF cDNA (4137bp) cloned into HindIII and BamHI sites of a pCMV vector.	Gift from Prof G Mandel.
HZ4	Contains 2kb of the human NRSF sequence, equivalent to the N-terminus domain, under the control of a CMV promoter (see figure 2.1)	Gift from Dr D.J.Anderson

bHLH plasmids		
pN3	pSG5 expression vector containing full length USF1	Gift from Dr M.Sawadogo
pN4	pSG5 expression vector containing full length USF2	Gift from Dr M.Sawadogo
Control Vectors		
pRL-TK-Renillin	Reporter gene construct, containing renillin luciferase DNA driven by the TK promoter. Used as an internal control	Promega

Table 2.1: Plasmids used in the current study. The above table summarises the plasmids used in this study and a brief overview of what they contain.

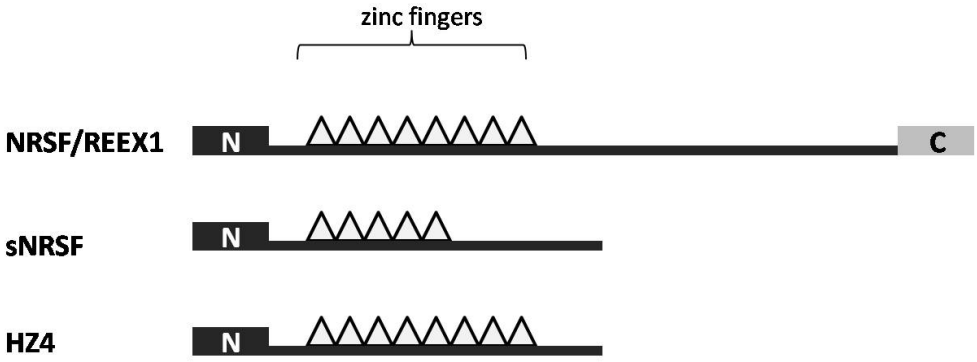

Figure 2.1. Schematic comparison of NRSF isoforms and the NRSF expression constructs employed. REEX1contains the full-length human NRSF cDNA, whilst HZ4 contains 2kb of human NRSF cDNA, incorporating the N-terminal domain and the eight zinc fingers found in full-length NRSF, but lacks the C-terminal domain. HZ4 is employed as a tool to mirror sNRSF over-expression due to the shared absence of the entire C-terminal domain, however it should be noted that human sNRSF contains only five out of eight zinc fingers, thus HZ4 only an analogous representation of sNRSF.

2.1.2 Commonly used solutions and reagents

2.1.2.1 Microbiology and Molecular Biology

Luria broth (LB) media

10.0g/l peptone, 5.0g/l yeast extract and 10.0g/l NaCl.

LB agar

10.0g/l peptone, 5.0g/l yeast extract and 12g/l agar.

5x TBE buffer

54g Tris(hydroxymethyl)methylamine (Tris) (BDH), 27.5g boric acid (VWR International), 20ml 0.5M Ethylenediamine Tetraacetic Acid (EDTA) (Sigma) and dH_2O up to 1 litre.

6x agarose gel loading buffer

Blue/Orange 6x loading dye (Promega)

DNA Ladders

Mass ruler (Fermentas), 100bp Ladder & 1Kb Ladder (both Promega)

ChIP fixation solution

20ml 1x PBS and 540µl 37% formaldehyde.

ChIP glycine stop-fix solution

2ml 10x Glycine (Active Motif), 2ml 10x PBS and 16ml dH_2O.

ChIP cell scraping solution

400µl 10x PBS, 3.6ml dH_2O and 10µl of 100mM PMSF added just prior to use.

Protein extraction buffer

50mM Tris-HCl pH 7.2, 150mM NaCl, 2mM EDTA, 1mM EGTA, 1% Triton x-100, 1 tablet of protease inhibitor cocktail (PICt) (Roche) 100 µM PMSF was added fresh from stocks before use.

1x NuPAGE MOPS SDS running buffer

760ml dH_2O and 40ml 20x NuPAGE MOPS SDS running buffer (Invitrogen).

20x NuPAGE MOPS SDS transfer buffer

500mM Bicine, 500mM Bis-Tris and 20.5mM EDTA to a volume of 125ml.

1x NuPAGE MOPS SDS transfer buffer

749ml dH_2O, 50ml 20x NuPAGE MOPS SDS transfer buffer, 200ml 100% molecular grade methanol (Sigma) and 1ml NuPAGE antioxidant. pH adjusted to pH7.2.

Triton-PBS

1% triton in PBS

Stripping buffer

100mM β-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl pH 6.7

2.1.3 Treatment solutions

2.1.3.1 Carbamazepine (CBZ)

CBZ (Sigma; Cat No: C4024) was dissolved in 100% molecular grade ethanol to make a 15mg/ml stock solution. This stock solution was diluted in complete media at a dilution of 1:300 or 1:1500, to make 50µg/ml or 10µg/ml, respectively.

2.1.3.2 Phenytoin (PHY)

PHY (Sigma; Cat No: D4007) was dissolved in 100% biotechnology performance grade dimethyl sulfoxide (DMSO) (Sigma; Cat No: D2438) to make a 20mg/ml stock solution. This stock solution was diluted in complete media at a dilution of 1:400 or 1:2000, to make 50µg/ml or 10µg/ml, respectively.

2.1.3.3 Lamotrigine (LMT)

LMT (Sigma; Cat No: L3791) was dissolved in 75% biotechnology performance grade DMSO (Sigma; Cat No: D2438) in ddH_2O to make a 5mg/ml stock solution. This stock solution was diluted in complete media at a dilution of 1:100 or 1:500, to make 50µg/ml or 10µg/ml, respectively.

2.1.3.4 Kainic Acid (KA)

KA (Sigma; Cat No: K0250) was dissolved in sterile ddH_2O to make a 1mM stock solution. This stock solution was diluted in complete media at a dilution of 1:200 or 1:1000, to make 50µM or 10µM, respectively.

2.1.3.5 Cocaine

Cocaine (Sigma; Cat No: C5776) was dissolved in sterile ddH_2O to make a 1mM stock. This stock solution was diluted 1:100 or 1:1000 in complete media, to make 10μM or 1μM, respectively.

2.1.3.6 Vehicle Control C

Vehicle control for CBZ treatment (Control C) was 100% molecular grade ethanol diluted in complete media at a ratio of 1:300, making a final concentration of 0.33% ethanol.

2.1.3.7 Vehicle Control P

Vehicle control for PHY treatment (Control P) was 100% biotechnology performance grade DMSO diluted in complete media at a ratio of 1:400, making a final concentration of 0.25% DMSO.

2.1.3.8 Vehicle Control L

Vehicle control for LMT treatment (Control L) was 75% biotechnology performance grade DMSO diluted in complete media at a ratio of 1:100, making a final concentration of 0.75% DMSO

2.1.3.9 Vehicle Control KA

Vehicle control for KA treatment was sterile ddH_2O diluted in complete media at a ratio of 1:200.

2.1.3.10 Vehicle Control Cocaine

Vehicle control for cocaine treatment was sterile ddH_2O diluted in complete media at a ratio of 1:100.

2.1.4 Cell culture media

2.1.4.1 Complete Media for human SK-N-AS neuroblastoma cell line

Dulbecco's modified eagle's media (DMEMs) (Sigma) supplemented with 10% Foetal bovine serum (FBS) (Perbio, Hyclone), 1% penicillin + streptomycin (Sigma), 1% non essential amino acids (NEAA) (Autogen Bioclear) and 1% 200mM L-glutamine (Sigma).

2.1.4.2 Serum Free Media for human SK-N-AS neuroblastoma cell line

DMEMs (Sigma) supplemented with 1% penicillin + streptomycin (Sigma), 1% NEAA (Autogen Bioclear) and 1% 200mM L-glutamine (Sigma).

2.1.4.3 Complete Media for human SH-SY5Y neuroblastoma cell line

1:1 ratio of DMEMs (Sigma) and Hams F12 (Sigma) supplemented with 10% FBS (Perbio, Hyclone), 1% penicillin + streptomycin (Sigma), 1% NEAA (Autogen Bioclear) and 1% 200mM L-glutamine (Sigma).

2.1.4.4 Serum Free Media for human SH-SY5Y neuroblastoma cell line

1:1 ratio of DMEMs (Sigma) and Hams F12 (Sigma) supplemented with 1% penicillin + streptomycin (Sigma), 1% NEAA (Autogen Bioclear) and 1% 200mM L-glutamine (Sigma).

2.2 Methods

2.2.1 General cloning methods

2.2.1.1 Polymerase Chain Reaction (PCR) primer design

Polymerase chain reaction (PCR) primers were designed using the online primer designer programmes Primer 3 (http://frodo.wi.mit.edu/) and NetPrimer (http://www.premierbiosoft.com/netprimer). Primer 3 was employed to generate a list of potential PCR primers to amplify a sequence of interest, and Net Primer was utilised to bioinformatically test the primers; screening for appropriate melting temperatures, GC% content, potential dimerisation and hairpin formation, rejecting primers whenever necessary. In general, primers were designed to be between 18-25 bp nucleotides long, have a melting temperature of 50-65 °C and a GC content between 40-60%. BLASTN searches were used to confirm the total gene specificity of the primer sequences chosen.

2.2.1.2 Standard PCR

PCR was employed to amplify specific DNA fragments for use in either molecular cloning applications, or to analyse mRNA expression of a specific gene. Standard PCR reactions were performed in a Px2 thermal cycler (Thermo Scientific). In general, for PCR reactions of DNA fragments used in molecular cloning, the proof reading polymerase enzyme *Pfu* DNA polymerase (Promega) was employed to amplify and proof read the DNA fragments. A 50µl *Pfu* PCR reaction included 10-100ng DNA template, 5µl *Pfu* reaction buffer with $MgSO_4$, 0.5µl 20pmol forward and 0.5µl 20pmol reverse primers, 1µl 10mM dNTPs and 0.75µl (1.25 unit) *Pfu* DNA polymerase (Promega). For standard PCR reactions, GoTaq polymerase (Promega) was employed.

25µl GoTaq PCR reactions included 10-100ng DNA template, 4µl 25mM $MgCl_2$, 5µl 5x GoTaq reaction buffer (Promega), 0.5µl 20pmol forward and 0.5µl 20pmol reverse primers, 1µl 10mM dNTPs and 0.2µl (1 unit) GoTaq polymerase (Promega). Specific Primers used throughout are given in Table 2.2.

2.2.1.3 PCR purification

To purify single- or double-stranded DNA fragments from PCR reactions or DNA from other reactions such as enzymatic digestion reactions, the QIAquick PCR Purification Kit (Qiagen) was used following manufactures' instructions. Briefly, 5x volumes of Buffer PB (Qiagen) to 1x volume of the PCR sample were mixed, applied into the QIAquick column and centrifuged for 1min at 13,000 rpm. The flow-through was discarded and the column was washed by adding 750µl PE buffer and centrifuged as before. Again, the flow-through was discarded and the column was re-centrifuged as before to completely remove residual Buffer PE. The column was then placed into a clean 1.5 ml eppendorf tube and the DNA was eluted using 30µl of Elution Buffer, incubated for 1min at room temperature (RT) and centrifuged as before.

Gene (Species)	Forward (5' – '3)	Reverse (5' – 3')	Product Size (bp)
Pol II (Human)	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA	267bp
NRSF (Human)	TATGCGTACTCATTCAGGTGAG	TTTGAAGTTGCTTCTATCTGCTGT	166bp
sNRSF (Human)	GGATACCATTTGGTAATATTTAC	TTTGAAGTTGCTTCTATCTGCTGT	191bp
USF1 (Human)	AACGTCAAGTACGTCTTCCGA	CCGTGCTGGGGAAGTAAGTAT	232bp
USF2 (Human)	AATTGATGGAACCAGAACACC	AAGCAGGGCGTTCTCATTCTT	313bp
TAC1 (Human)	ACTGTCCGTCGCAAAATCC	TCCAAAGAACTGCTGAGGC	220bp
NKB (Human)	TGCTGCTATTCACAGCCATC	AGCAATCCCTCCAGAGATGA	174bp
HCN2 (Human)*	CGTTACCAGGGCAAGATGTTTG	GTTGTCCACGCTCAGCGAAT	393bp
HCN3 (Human)**	CCTACAGCGACTTCCGGTTT	GTGCCGTTTTGTAGACCTCAGC	351bp
SCN9a (Human)	GAGGCCTGTTTCACAGATGG	TGGGCCAAGATCTGAGTAG	401bp
CART (Human)	CAGCAACGACGAGTTTCAGA	TGGGGATGTATGGAGGAGAA	405bp
β-actin (Human)	CATCCTCACCCTGAAGTACC	ATAGCAACGTACATGGCTGG	219bp

Table 2.2.1 Primers used in RT-PCR and qPCR. *HCN2 primers from Stilitano *et al*., 2008. ** HCN3 primers from Varghese *et al*., 2006. *** HDAC2 primers from Tateno *et al*., 2007.

DNA fragment	Forward (5' – '3)	Reverse (5' – 3')	Product Size (bp)
pNKB-757 (-757 to +181)	AAACGCGTCTCGTGAAACTCCAC AACGA	AACTCGAGCATCCAGCATTCTCC CACTT	954bp
pNKB-289 (-289 to +181)	AAACGCGTCTTCTGAGGCGGCTA TTGAG	AACTCGAGCATCCAGCATTCTCC CACTT	358bp

Table 2.2 Primers used in molecular cloning, for amplification of the Human NKB proximal promoter.

2.2.1.4 Analysis of DNA using Agarose Gel-Electrophoresis

To analyse DNA fragments from PCR reactions or from restriction digests, agarose gel-electrophoresis was employed. 1-2% agarose gels were made by dissolving 1-2% multi-purpose agarose (Bioline) in 0.5x TBE buffer, heated in a microwave until boiling. The resulting solution was supplemented with 5µl ethidium bromide (10mg/ml aqueous solution, Sigma E-5134) once cooled. Gels of 60ml or 100ml were cast in 12x14cm or 20.5x10cm trays, respectively and the appropriate combs were inserted. Gels were left at RT until set and were then submerged in horizontal gel electrophoresis tanks (Hybaid turn and cast submarine gel system, Hybaid, or Savant HG 350 tank) containing 0.5x TBE buffer. All samples beside GoTaq PCR reaction samples were supplemented with 6x Blue/Orange loading buffer (Promega) and loaded into the wells. GoTaq PCR reactions contained loading dye in the GoTaq reaction buffer, and hence did not require addition loading buffer. The size of a PCR product or restriction digest fragments was determined by loading a DNA ladder (Mass ruler: Fermentas; 100bp Ladder: Promega; 1Kb Ladder: Promega). Gels were run for 1 hour at 100Volts (Hybaid). The electrophoretically separated DNA was then visualised using an Evenscan broadband dual wavelength transluminator in a MultiImageII Light Cabinet (both Alpha Innotech Corporation) at a wavelength of 302nm. Images were recorded with a CCD camera (Alpha Innotech Corporation) and stored electronically.

2.2.1.5 Recovery of DNA from agarose-gels

DNA products from PCR reactions or restriction digests were run and separated using agarose gels. In cases where the DNA was required, the DNA had to be recovered

from the agarose gels. The QIAquick Gel Extraction Kit (Qiagen) was used to extract and purify the DNA out of the agarose gels, following manufacturers' instructions. Briefly, the desired DNA band was excised from the gels, under UV translumination, using a clean blade and the resulting gel slices were weighed. To dissolve the agarose gel, 3x volumes of buffer QG was added to 1 volume of gel slice, and incubated for 10mins at 50°C. 1x gel volume of 100% molecular grade isopropanol (Sigma) was added to each sample and vortexed to mix. This was then applied into a QIAquick spin column, placed within a 2ml collection tube and centrifuged for 1min at 13,000rpm using a bench top centrifuge. The flow-through was discarded and the column was washed by adding 750µl PE buffer and centrifuged as before. The flow-through was then discarded and the column was re-centrifuged as before to completely remove residual buffer PE. The column was then placed into a clean 1.5ml eppendorf tube and the DNA was eluted using 30µl Elution Buffer, incubated for 1 minute at RT and centrifuged as before.

2.2.1.6 Generation of NKB promoter reporter gene constructs

Two human NKB promoter reporter gene constructs were generated as follows: DNA fragments of the NKB proximal promoter region spanning either -757 to +181 (pNKB-757) or -289 to +181 (pNKB-289), were PCR amplified from human genomic DNA (Promega), using the *Pfu* DNA polymerase enzyme (Promega). Primers used to amplify each DNA fragment (Table 2.2.2) were designed with flanking MluI (forward primer) and XhoI (reverse primer) restriction enzyme sites for directional cloning into the corresponding MluI and XhoI sites in pGL3basic (Promega) promoter-less luciferase reporter gene construct (upstream of the luciferase reporter gene). Furthermore, an

additional AA sequence was added 5' of each primer to enhance cleavage efficiency. The PCR reaction conditions were as follows: denaturation at 95^{o}C for 2mins, followed by 40 cycles of: 95^{o}C for 30 seconds, 60^{o}C for 30 seconds, 72^{o}C for 1mins, followed by a final elongation step 72^{o}C for 5mins. Resulting DNA was ran on an agarose gel (Section 2.2.1.4), gel extracted and purified (Section 2.2.1.5).

The DNA fragments and the target pGL3basic vector were then subjected to restriction enzyme digestion, to generate the MluI and XhoI sticky ends required for ligation. Briefly, 8µl of DNA (PCR product or pGL3basic) was mixed with 1µl 10x reaction buffer D, 0.5µl MluI and 0.5µl XhoI (all Promega), and incubated overnight at 37^{o}C. The resulting cleaved DNA was purified using the Qiagen PCR purification protocol (See section 2.2.1.3) to remove enzymes. The purified NKB promoter fragments were then ligated into pGL3basic (Section 2.2.1.7).

2.2.1.7 Ligation

In order to ligate the NKB promoter DNA fragments into the pGL3basic vector, the amount of DNA required needed to be calculated, via the the following ligation calculation: The amount of insert required (ng) =

[(ng vector X insert size kb)/(vector size kb)] X insert:vector ratio = ng insert

In general, two different insert:vector molar ratios were used, either 1:1 or 3:1 ratios. In the ligation reaction 1µl (25ng) of vector and appropriate volume of insert were added to 1µl 10x ligase buffer (NEB) and 1µl 200 units of T_4 DNA ligase (NEB) in at total volume of 10µl, incubated at RT for 4 hours then at 4˚C overnight.

2.2.1.8 Transformation of chemically competent E. Coli cells

Following ligation of the NKB promoter into the pGL3basic vector, the resulting plasmid was transformed into strains of chemically competent *E. Coli* DH5-α cells (Invitrogen), following manufacturers guidelines. Briefly, the ligation reaction (10µl) or 10ng DNA (for transformation of other DNA) were added to 50µl of competent DH5-α cells and incubated on ice for 30mins. The cells were then subjected to 'heat-shock' in a water bath (at 42^{o}C) for 45 seconds and then incubated on ice for 2mins. Following this, 950µl of pre-warmed LB broth was added to the cells, and the culture incubated on a shaker (at 225rpm), at 37^{o}C for 1 hour. Between 50-200µl of this culture was spread evenly onto LB agar plates supplemented with 100µg/ml ampicillin, and cultured overnight at 37˚C.

2.2.1.9 Isolation of DNA constructs from bacteria

2.2.1.9.1 Mini-preparation of plasmid DNA

In order to test plasmids following molecular cloning manipulation, a small scale preparation of DNA (up to 20µg) was undertaken. To test colonies grown on LB agar plates, individual colonies were transferred to 5ml LB broth supplemented with 100µg/ml ampicillin, and cultured overnight at 37^{o}C, on a shaker (at 225rpm). DNA was isolated from the resulting bacterial culture using the QIAprep Spin Miniprep Kit (Qiagen), according to manufacturer's guidelines. Briefly, the bacterial culture was pelleted following centrifugation at 4000rpm for 5-10mins, and resuspended in a resuspension buffer. The cells were lysed using a modified alkaline lysis buffer for 5mins at RT, following which the lysis was neutralised and adjusted to high salt binding

conditions, using a neurtralisation buffer. The neutralised lysate was cleared by centrifugation (13,000rpm for 10mins) before being applied into a column containing a membrane that selectively absorbs DNA in high-salt conditions. The column was then washed with buffer PB to remove endonucleases and then salts were removed by a final wash with buffer PE (both Qiagen). The plasmid DNA was then eluted into a fresh 1.5ml eppendorf using 50µl nuclease-free water. This plasmid DNA was next tested by either using restriction enzyme digestion assays (Section 2.2.1.11) or sent for DNA sequencing (2.2.1.12).

2.2.1.9.2 Maxi-preparation of plasmid DNA

For large scale production and isolation of DNA (up to 500µg), the QIAGEN Plasmid Maxi Kit (Qiagen) was employed. As with mini-preparation (Section 2.2.1.9.1), a bacterial culture is required, however this was increased from a 5ml overnight culture, to a 100ml overnight culture. Briefly, the 100ml culture was pelleted by splitting the culture into two 50ml falcon tubes, and centrifuged for 15mins at 4500rpm at 4^{o}C. The resulting pellet was resuspended in the provided resuspension buffer supplemented with RNase A, and the cells were lysed at RT for 5mins, using a modified alkaline lysis buffer. The lysis reaction was neutralised following the addition of a potassium acetate neutralisation buffer, incubated on ice for 20mins. The resulting cell lysate contained a mixture of plasmid DNA, bacterial protein, DNA and cell debris. This cellular debris was removed via centrifugation at 13,000 rpm for 45mins, the supernatant was passed through a QIAfilter Maxi Cartridge and the DNA was bound to an anion-exchange column under high salt and low pH conditions. Medium-salt washes were used to remove RNA, proteins etc, and the plasmid DNA was eluted with a high-salt wash. The

eluted DNA was then precipitated with 0.7 volumes 100% molecular grade isopropanol (Sigma) and centrifuged for 30mins at 13,000rpm. The resulting DNA pellet was washed with 70% molecular grade ethanol (Sigma) and re-centrifuged a further 10mins at 13,000rpm. The DNA was resuspended in 100µl nuclease-free water and transferred into a 1.5ml eppendorf for storage at -20^{o}C.

2.2.1.10 Analytical restriction enzyme digests

To test DNA fragments and DNA plasmids used in molecular cloning, or to screen for positive colonies following a ligation and transformation reaction, restriction enzyme digestion was employed. Restriction enzyme digests were carried out in an appropriate 1x restriction enzyme buffer. All restriction digest reactions were undertaken for a minimum time of 3 hours, incubated at the appropriate temperature for the specific restriction enzyme. In general, multiple restriction enzymes could be used in a single reaction, if the enzymes used a common reaction buffer. In cases where the restriction enzymes required different reaction buffers, a double digest would occur. Two or more separate digestion reactions would be performed, beginning with the enzyme that functioned in the reaction buffer with the lowest salt concentration. The following reaction would utilise the enzyme that functioned in the reaction buffer with the next lowest salt concentration, and so on. Each digest would be adjusted to maintain the ratio of 1:10, reaction buffer:total volume. Resulting DNA fragments were visualized after gel electrophoresis in a UV light transilluminator (Section 2.2.1.4).

2.2.1.11 Sequencing

DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big-Dye Ver3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

2.2.1.12 Measurement of DNA/RNA concentration by spectrophotometry

DNA and RNA concentrations were determined using an UV spectrophotometer (Jenway Genova Life Science Analyser). The UV spectrophotometer was zeroed against 100µl dH_2O before 1:20 diluted DNA or RNA preparation was placed into a quartz cuvette and placed in the cell holder for the determination of concentration, using the following formulae:

dsDNA concentration = OD_{260} value (at wavelength of 260 nm) x 50 x dilution factor

ssDNA concentration = OD_{260} value (at wavelength of 260 nm) x 33 x dilution factor

RNA concentration = OD_{260} value (at wavelength of 260 nm) x 40 x dilution factor

where 1 OD at 260 nm equals 50ng/µl of dsDNA (double-stranded DNA), 33ng/µl of ssDNA (single-strandard DNA) and 40ng/µl of RNA, hence x50, x33and x40 respectively.

2.2.2 Cell Treatment & Transfection

2.2.2.1 Cell Culture

2.2.2.1.1 Culture of SK-N-AS cells

Human neuroblastoma SK-N-AS cells were obtained from American Type Culture Collection (ATCC) and maintained as an adherent monolayer in DMEMs medium (Sigma) as described in section 2.1.4. SK-N-AS cells were cultured at 37^{o}C, 5% CO_2, in T75 flasks and the culture media was routinely changed every other day. Cells were split when 70−80% confluent.

2.2.2.1.2 Culture of SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were also obtained from ATCC and maintained as an adherent monolayer in a 1:1 ratio EMEMs: HAM's F12 medium (both Sigma) as described in section 2.1.4. As with SK-N-AS cells, SH-SY5Y cells were cultured at 37^{o}C, 5% CO_2, in T75 flasks and the culture media was routinely changed every other day. Cells were split when 70−80% confluent.

2.2.2.2 Cell Treatment

SK-N-AS cells and SH-SY5Y cells were seeded into 24-wells plates (luciferase assays), 12-well plates containing sterile coverslips (immunofluorescence), 6-well plates (Endogenous gene expression assays) or T175flasks (Chromatin Immunoprecipitation (ChIP) assays), at a density of 0.5x10^5 cells/cm^2, and cultured until 70% confluency was achieved. Prior to drug or control treatment, media was removed and replaced with corresponding serum free media (serum starvation) and left overnight.

Cells were subsequently incubated in corresponding complete media supplement with either: KA (at 1µM or 5µM), CBZ (at 10µg/ml or 50µg/ml), PHY (at 10µg/ml or 50µg/ml), LMT (at 10µg/ml or 50µg/ml), cocaine (at 1µM or 10µM), or corresponding vehicle controls (See section 2.1.3), for either 4hrs or 24hrs. Following treatment, the cells were immediately harvested, allowing no recovery period. Cells treated with these drugs were used in a range of assays, including transfections, endogenous gene expression assays, immunofluorescence and ChIP as described below.

2.2.2.3 Delivery of luciferase constructs into neuroblastoma cell line

Reporter gene plasmids (Table 2.1) were delivered into human SK-N-AS neuroblastoma cells using ExGen 500 *in vitro* Transfection Reagent (Fermentas). To normalise for transfection efficiency a modified pMLuc-2 vector containing a minimal TK promoter followed by an optimized Firefly luciferase cDNA were transfected into the cells to act as an internal control. The internal control was delivered at a ratio of 1:50 ration pMLuc-2: reporter gene construct.

2.2.2.4 ExGen 500 *in vitro* Transfection

ExGen 500 *in vitro* Transfection Reagent (Fermentas) was used to transfect human SK-N-AS neuroblastoma cells. ExGen 500 is a polyethylenimine cationic polymer which interacts with DNA to form small, stable, highly diffusible particles that settle on the cell surface. The DNA/ExGen 500 complex is then absorbed into the cell via endocytosis. The resulting endosomes are then ruptured within the cytoplasm before lysosomal degradation releases the DNA/ExGen 500 complex, allowing the DNA to be translocated into the nucleus. Following manufactures' instructions, 1µg of reporter

constructs and 20ng of the internal control plasmid pmLuc-2 were diluted in 100µl of 150mM NaCl, vortexed and brief centrifuged. 3.3µl of ExGen 500 was added per 1µg of DNA used. The solution was immediately vortexed for 10 seconds and incubated for 10mins at RT. 100µl of the DNA/ExGen 500 mixture was added to each well, so that the resulting volume of the DNA/ExGen 500 mixture equalled 10% of the total volume of the culture medium. The plates were then centrifuged for 5mins at 280xg and finally incubated at 37°C for 48 hours in a humidified 5% CO_2 incubator. 48 hours post-transfection the cells were lysed to analyse transgene expression.

2.2.2.5 Co-transfection experiments

To assess the potential regulation of the human NKB proximal promoter region or the rodent TAC1 promoter region, by particular TFs, constructs expressing full length NRSF (RE-EX1), a truncated version (HZ4), USF1 (pN3) and USF2 (pN4) proteins were transfected into cell lines simultaneously with the reporter constructs (Table 2.1). The constructs were co-transfected using ExGen 500 following the protocol described above (section 2.2.2.2). In co-transfection experiments the amount of plasmid DNA transfected into the cells was maintained constant. For this, per every 1µg of expression vectors co-transfected with reporter constructs, an equal amount of innocuous DNA (pGL3b) was transfected with the reporter gene constructs when basal levels of transcription were assessed (no expression construct present).

2.2.2.6 Analysis of transgene expression by Reporter gene assay

Analysis of the amount of luciferase protein activity produced by the transfected plasmids was estimated using the Dual Luciferase Assay kit (Promega, Madison Cat. No

E1500) on extracts of transfected cells. Briefly, cell extracts were obtained as follows: culture medium was removed and wells were washed twice with PBS. 70µl of 1x passive lysis buffer per well was added and incubated for 15mins on a rocking platform. At the end of this period, 20µl supernatant containing the lysed cell extracts, were plated into a 96-well plate and transferred into the Glomax 96 microplate luminometer (Promega). 100µl Firefly luciferase reagent and 100µl Renillin luciferase reagent were automatically dispensed into each well to calculate luminescence intensity. The Renillin luciferase substrate solution was added to each sample to determine the protein production of the internal control (pmLuc-2) to normalise for transfection efficiency in case the number of cells or the efficiency of the transfection varied from well to well. The calculated luminescence was processed by the Glomax 96 software, and data transferred and analysed in Microsoft Excel.

2.2.3 mRNA analysis

2.2.3.1 RNA extraction

To analyse gene expression in treated and untreated cells, SK-N-AS and SH-SY5Y cells were plated out and cultured on sterile 6-well plates. Cells were grown until 70% confluent, and treated as described in section 2.2.2.2. Treated or untreated cells were harvested and RNA extracted using TRIzol (Invitrogen), according to manufacturer's guidelines. Briefly, 1ml TRIzol was added to each well and incubated for 5mins at RT to permit the complete dissociation of nucleoprotein complexes. The TRIzol was then transferred to 1.5ml eppendorfs, and 200µl of choloform solution was added to separate RNA from DNA and protein, through phase separation. Samples were

mixed vigorously for 15 seconds and incubated for 2-3mins at RT. Samples were then centrifuged at 12,000x *g* for 15mins at 2-8°C. Following centrifugation the mixture separated into lower red, phenol-chloroform phase (an interphase) and a colourless upper aqueous phase. The RNA containing upper aqueous phase was transferred into fresh RNase-free 1.5ml eppendorf tubes and 500µl 100% molecular grade isopropanol (Sigma) was added. The mixture was incubated for 10mins at RT and then centrifuged at 12,000x *g* for 10mins, at 2-8°C, to precipitate the RNA. The resulting RNA pellet was washed with 70% molecular grade ethanol, and re-centrifuged for 5mins at 7,500x *g*, at 2-8°C. The RNA pellet was then left to air-dry for 5-10mins, allowing complete evaporation of any residual ethanol. To dissolve the RNA, the pellet was resuspended in 50µl of RNase free water and incubated for 10mins at 55-60°C. The RNA samples were stored at -80˚C. RNA concentration was determined as described in section 2.2.1.13.

2.2.3.2 DNase digestion of total RNA extraction

In order to remove any, potentially contaminating, DNA from the harvested RNA, each RNA sample was digested with RQ1 RNase-free DNase (Promega). Briefly, 1µg of total RNA was digested with 1µl (1unit) RQ1 RNase-free DNase supplemented with 1µl RQ1 10x buffer (Promega), in a total 10µl reaction volume. The reaction was incubated at 37^{o}C for 30mins. To stop the reaction, 1µl Stop-solution (Promega) was added, and the DNase enzyme was inactivated at 65^{o}C for 10mins.

2.2.3.3 Reverse Transcriptase PCR (RT-PCR)

For conversion of RNA to complementary DNA (cDNA), the Reverse Transcription System (Promega) was utilised, according to manufacturer's guidelines.

Briefly, 1µg DNase treated RNA was used as a template in a 20µl reaction containing: 1µg of oligo dT primers, 2µl x10 Reverse Transcriptase reaction buffer, 2µl 10mM dNTPs, 0.5µl RNAsin ribonuclease inhibitor, 4µl 25mM $MgCl_2$, 0.75µl reverse transcriptase (all Promega) plus dH_2O to take the volume up to 20µl. For first strand cDNA synthesis, the reverse transcription reaction was carried out at 42°C for 15mins for annealing, followed by 95^oC for 5mins to inactivate the reverse transcriptase. The resulting cDNA concentration was determined (as described in section 2.2.1.13), diluted to 10ng/µl, for use in RT-PCR and quantitative PCR (qPCR), and stored at -20^oC.

2.2.3.4 PCR to analyse mRNA expression

To analyse mRNA expression of specific genes, PCR was employed. PCR reactions were performed in a Px2 thermal cycler (Thermo Scientific), using GoTaq polymerase (Promega). Primers used throughout are given in Table 2.2.1, and all were obtained from MWG Biotech (Germany), prepared as a 100µM stock and stored at -20˚C.For each 25µl PCR reaction, 10ng cDNA template was used, in combination with: 4µl 25mM $MgCl_2$, 5µl GoTaq reaction buffer (Promega), 0.5µl 20pmol forward and 0.5µl 20pmol reverse primers, 1µl 10mM dNTPs (Promega) and 0.2µl (1 unit) GoTaq polymerase (Promega). DNase-free and nuclease-free dH_2O was used to make a total volume of 25µl.

PCR thermal cycle conditions were adapted for each gene investigated, with the annealing temperature varying according to the melting temperature of the primer pairs used and the extension time adapted according to the size of the expected product, with 1 minute extension time for each 1000bp employed as a guide. In general, the thermal

cycle conditions were as follows: initial denaturation: 95^{o}C for 2mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds, annealing 60-62^{o}C for 30 seconds and elongation: 72^{o}C for 1min; and a final elongation step of 72^{O}C for 2mins.

2.2.3.5 Quantitative PCR (qPCR)

For more accurate analysis of mRNA expression, quantitative PCR (qPCR) was employed. qPCR reactions were performed using the iQ SYBR Green supermix (BioRad) in a BioRad iQ5 thermal cycler. The SYBR green method works via the binding of the SYBR green I fluorescent dye to DNA, which becomes excited in the presence of dsDNA generating fluorescence. The levels of fluorescence are directly proportional to the copy number of amplicons produced, and hence the quantity of PCR product can be measured, according to levels of fluorescence. The specificity of qPCR products can be determined by subsequent melt curve analysis, from 55°C to 95°C with 0.5°C increments. The content of unknown samples can be calculated from the amount of the target gene, normalised relative to the amount of the housekeeping gene RNA polymerase II (Pol-II), using the 'Relative Quantification' calculation of $2^{-\Delta\Delta C}{}_{T}$ (Livak & Schmittgen., 2001). This equation takes into account the cycle threshold (CT) (the cycle number when the levels of recorded fluorescence meet a predetermined threshold level) of a target gene versus a house keeping control gene, and derives the initial quantity of that gene, based on the assumption PCR amplification if perfectly exponential.

Each 25µl qPCR reactions contained 10ng cDNA as template, 12.5µl iQ SYBR Green master mix (containing iTaq hot-start DNA polymerase, SYBR Green I dye, optimized PCR buffer, 5mM $MgCl_2$, dNTP mix including dUTP and fluoresein), 0.3µl

20pmol forward and 0.3µl 20pmol reverse primers and DNase-free and nuclease-free dH2O to bring the total volume to 25µl. qPCR thermal cycle conditions for all genes (except the truncated NRSF isoform sNRSF) were as follows: initial denaturation step: 95°C for 3mins for 1 cycle (activates the iTaq DNA polymerase enzyme), followed by 40 cycles of denaturation: 95°C for 30secs and annealing: 62°C for 30secs. For sNRSF, the conditions were modified to: 95°C for 3 min for 1 cycle; 40 cycles of denaturation: 95°C for 30secs and annealing: 58°C for 45secs.Continuous fluorescence was detected using the iQ5 thermal cycler software.

2.2.3.6 Affymetrix Microarray Analysis

In order to analyse global changes in gene expression patterns, Affymetrix Microarray technology was utilised. A total of 10µg of RNA was processed using established Affymetrix protocols for the generation of biotin-labeled cRNA and the hybridization, staining, and scanning of arrays as outlined in the Affymetrix technical manuals (Van Gelder *et al*., 1990; Warrington *et al*., 2000). The processed RNA was hybridized to Human Genome U133 Plus 2.0 arrays from Affymetrix, Inc. (Santa Clara, CA), and scanned on an Affymetrix GeneChip® scanner 3000 at 2.5µm resolution, and image data was analysed using Affymetrix GCOS software. A more complete description of this process is available in Dobbin *et al*., 2005.

Samples were compared pairwise to corresponding controls. The data generated for each comparison was filtered for (a) Fold-change smaller or equal to 0.5, or greater or equal to 2.0 representing a decrease of increase in expression respectively, (b) p-value

change smaller than 0.05 or greater than 0.95 (two-sided p-value) and (c) Gene detected as present (P) or moderately present (M) in at least one of the two samples.

2.2.4 Protein Analysis

2.2.4.1 Protein Extraction from Cell Lysates

In order to extract protein from cell lysates, cell pellets were washed with 1xPBS and lysed in 1ml protein extraction buffer (See section 2.1.2.1) in a 1.5ml eppendorf tube, for 30mins on ice. The pellet was then passed through a syringe x10 times, and cellular debris was removed through centrifugation, at 13,000rpm, for 15mins at 4^{o}C. The supernatant containing the protein extract was stored at -80˚C.

2.2.4.2 Determination of protein concentration

In order to perform western blotting, the concentration of protein in each extract was first determined using a BCA Protein Assay Kit (Pierce). This assay is based on the chemical reduction of Cu^{2+} ions to Cu^{+} ions by proteins under alkaline conditions. Each Cu^{+} ion then reacts with two bicinchoninic acid (BCA) molecules and generates a purple product. This purple product can be used to determine the protein concentration of the unknown sample, based on a comparison of the purple intensity against the intensities generated from a set of standards with known protein concentrations. In short, a set of standards with protein concentrations ranging from 2000µg/ml to 25µg/ml were made up. 25µl of each standard and sample were pipetted into a clear 96-well microplate, and 200µl of working reagent (made up from reagents A and B at a 50:1 ratio) were added. The reaction was incubated for 30mins at 37˚C. The plate was then cooled to RT and read in a plate reader (Lucy-2 microplate luminometer, Anthos Labtec Instruments

GmbH) at a wavelength of 570nm. The protein concentration was quantified using Microsoft Excel.

2.2.4.3 Western Blotting

2.2.4.4.1 Denaturing SDS-PAGE

For protein separation, NuPAGE 4-12% gradient of 10% Bis-Tris SDS-PAGE precast gels were used together with the XCell *SureLock* Mini-Cell system (Invitrogen). The gels were submerged in the inner chamber with 200ml 1x NuPAGE MOPS SDS running buffer (Section 2.1.2.1) containing 500µl NuPAGE antioxidant (Invitrogen). The outer chamber was then filled with 600ml 1x NuPAGE MOPS SDS running buffer.

20µg of either SK-N-AS or SH-SY5Y cellular protein extracts were mixed with 1x NuPAGE LDS sample buffer and 0.2x 1M DTT, and boiled for 10 minutes, before being loaded into the gel. For identification of molecular weight of the separated proteins SeeBlue Plus2 Prestained Standard 1x protein ladder (Invitrogen) was loaded. Samples and markers were separated at a constant voltage of 200V for 1 hour.

2.2.4.4.2 Electrophoretic transfer

Following gel electrophoresis, the gel apparatus was dismantled, and the gels were submerged in 1x NuPAGE transfer buffer (Section 2.1.1.2). To transfer proteins from the SDS-PAGE gels onto nitrocellulose membrane, the BioRad Mini-Protein II cell system was employed. Sponges, filter paper and nitrocellulose membrane (BioTrace®NT, Pall Life Sciences),were all also soaked in 1x NuPAGE transfer buffer, and a transfer sandwich was assembled in transfer cassettes, according to the manufacturer's guidelines. The cassettes were inserted into the BioRad transfer

apparatus, together with a frozen cooling unit to ensure overheating did not occur, and transferred for 1hour at 60V. After transfer, the transfer apparatus was disassembled and the nitrocellulose membrane was stained for 1min in Ponceau-S solution (Sigma), and washed in water. Staining of the transferred protein on the nitrocellulose membrane served as a control for an efficient and even transfer.

2.2.4.4.3 Detection of protein

For the removal of non-specific binding, the nitrocellulose membrane was blocked in 5% dry semi-skimmed milk/ 0.1% Triton/PBS overnight at 4°C. The nitrocellulose membrane was incubated with primary antibodies in PBS-T plus 2% milk at a 1 in 1000 dilution, on a rocker for 1 hour at RT or overnight depending on the antibody. NRSF and sNRSF antibodies were a kind gift from Noel Buckley, King's College London. The NRSF antibody was raised against the rat C-terminal sequence CYFLEEAAEEQE. The antibody against the truncated isoform, was raised against an alternative rat C-terminal epitope SGCDLAG (Belyaev *et al.*, 2004). Both anti-sera were raised in rabbit. These antibodies have been widely used in ChIP assays, for detection of protein by Western and immunofluorescence and in gel-shift experiments (Wood *et al*, 2003, Belyaev *et.al.,* 2004 and Spencer *et al.,* 2006).

The USF1 antibody is a rabbit polyclonal antibody raised against an epitope in the C-terminus of the human USF1 protein (Santa Cruz, Biotechnology). The USF2 antibody is a rabbit polyclonal antibody raised against an epitope in the N-terminus of the mouse USF2 protein (Santa Cruz, Biotechnology). Both antibodies have been used

in western blotting, immunofluorescence and ChIP (Park *et al.,* 2008; Rada-Iglesias *et al*., 2008; Crusselle-Davis *et al*., 2006).

After primary antibody incubation, the membrane was washed 3x for 10mins in 0.1% Triton/PBS, and then incubated in horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (GE Healthcare) at RT for 1 hour (at 1 in 2000 dilution). This was followed by washing as before. Enhanced chemiluminescence (ECL) was employed to visualize the antibodies, via the ECL Plus Western Blotting Detection Reagent (Amersham Biosciences) and Hyperfilm ECL (Amersham Biosciences), following manufacturers guidelines.

2.2.4.4.4 Stripping and re-probing of a Western Blot

Western blots can be stripped of signal and antibodies, and re-probed with a different antibody. The blot was submerged in stripping buffer (section 2.1.1.2) for 1 hour with gently agitation. The blot was then washed x6 10mins in PBS. The re-probing followed the standard western protocol, starting with the blocking step.

2.2.4.4 Immunofluorescence

Human SH-SY5Y cells plated glass coverslips were washed x3 in PBS and fixed in 4% paraformaldehyde (PFA) for 15mins at RT. The cells were washed as before, and permeabilised by incubation in 0.1% Triton/PBS for 10mins, RT. The cells were washed as before, and blocked for a minimum of 1hr with 2% BSA in PBS. For detection of NRSF, the truncated isoform, USF1 or USF2, the corresponding primary antibody was diluted 1 in 100m in 2% BSA in PBS, added to the coverslip, and incubated overnight at 4^{o}C. Cells were washed as before, and then incubated with Alexa Fluor 568 anti-rabbit

secondary antibody (Invitrogen), diluted at 1:200 in 2% BSA in PBS, for 1hr, RT . As a negative control, cells were stained with secondary antibody alone. Cells were washed in PBS as before, and coverslips were mounted onto slides in DAPI containing Vecta-Shield (Vector Laboratories), and sealed with nail varnish. Staining of the proteins was viewed using two photon confocal zeiss LSM S10 microscopy and excitation at wavelength 568 (Alexa 568) and infra red (DAPI).

Antibody	Clonality	Host Species	Immunogen	Source
anti-NRSF	Polyclonal	Rabbit	CYFLEEAAEEQE	Gift from Prof. N. Buckley
anti-REST4	Polyclonal	Rabbit	SGCDLAG	Gift from Prof. N. Buckley
anti-USF1	Polyclonal	Rabbit	Against C-terminus of human USF1	Santa Cruz Biotechnology
anti-USF2	Polyclonal	Rabbit	Against N-terminus of mouse USF2	Santa Cruz Biotechnology
IgG Control	Polyclonal	mouse	Against mouse IgG	Active Motif

Table 2.3. Antibodies used for western blotting , immunofluorescence and ChIP. Antibodies used for exclusively in ChIP are highlighted with orange shading.

2.2.5 Chromatin Immunoprecipitation (ChIP)

2.2.5.1 Cell Fixation and Chromatin Isolation

Human SH-SY5Y cells or SK-N-AS cells were grown to 70-80% confluency in T175 culture flasks prior to treatment or harvest. When ready for harvest, cells were washed twice with 10x PBS, and DNA-protein interactions were cross-linked (fixed) with Fixation solution (See section 2.1), and incubated for 10mins at RT on a rocking

platform. Following fixation, the cells were briefly washed with chilled 10x PBS, before the cross-linked interactions were quenched, with the addition of 10mls Glycine Stop-Fix solution (See section 2.1) and incubated on a rocking platform for 5mins. Cells were washed as before, and then scraped away from the culture plate, under 2mls cell-scraping solution (See section 2.1), using a cell scraper. Cells were then transferred to 15ml conical tubes and centrifuged for 10mins at 2,500 rpm at 4 °C, to pellet the cells. After discarding the supernatant, the cell pellet was re-suspended in 1ml ice-cold Lysis Buffer supplemented with 5µl PICt and 5µl PMSF (ensuring the DNA-protein interactions were preserved during chromatin purification and immunoprecipitation (IP)) (Active Motif) and incubated for 30mins on ice. To disrupt the cells and release the nuclei, the cells were passed through a needle/syringe ~5-10 times. The resulting solution was transferred to a 1.5ml eppendorf tube and centrifuged for 10mins at 5,000 rpm, at 4 °C to pellet the nuclei. The supernatant was carefully removed and discarded. The nuclei pellet was then resuspended in 1ml Shearing Buffer supplement with 5µl PICt (both Active Motif) and distributed equally into three 1.5ml eppendorf tubes.

2.2.5.2 Chromatin Sonication Shearing

Chromatin was sheared by sonication; specifically 20x 30seconds pulses at 50% power using a Microson sonicator (Misonix), with each of the triplicate 1.5ml eppendorfs maintained on ice, to prevent over-heating. After each pulse, the sheared chromatin samples were allowed to cool on ice for 1min before being centrifuged at 15,000 rpm at 4 °C for 1min. After the required number of pulses (20), the sheared chromatin was centrifuged at 15,000 rpm at 4°C for 12mins. The resulting supernatants

from each 1.5nl eppendorf, containing the sheared chromatin, was pooled into a fresh tube and stored at -80^{o}C.

2.2.5.3 Estimate shearing efficiency and Chromatin concentration

In order to determine the shearing efficiency of the sonication procedure, 50µl of sheared chromatin sample was transferred to a fresh 1.5ml eppendorf tube. To this, 150µl dH_2O, 8µl 5M NaCl and 1µl RNase was added to reverse cross-links and the sample mix was vortexed and incubated at 65^{o}C for 4hrs. Following incubation, the tubes were briefly centrifuged and allowed to return to RT. 10µl of Proteinase K (Active Motif) was added and the mixture was incubated at 42^{o}C for 1.5 hours. To Determine the DNA concentration, the DNA was phenol/chloroform extracted and precipitated by adding 200µl phenol/chloroform to the sample, vortexed and centrifuged for 5mins at 13,000rpm. The DNA containing aqueous top layer was then transferred to a fresh 1.5ml eppendorf tube containing 20µl 3M Sodium Acetate (pH 5.2) and 500µl 100% ethanol. The sample was vortexed to mix completely and placed at -20^{o}C for at least 1 hour. Following incubation, the sample was centrifuged at 13,000rpm for 10mins at 4°C. The supernatant was discarded carefully so not to disturb the pellet. The pellet was then washed with 500µl 70% ethanol, and re-centrifuged at 13,000rpm for 5mins at 4°C. After discarding the supernatant, the pellet was allowed to air-dry for 5-10mins at RT, and then the pellet was re-suspended in 50µl dH_2O. The DNA concentration was determined as described in section 2.2.1.12. The shearing efficiency of the sonication was determined by running 10µl DNA sample out via agarose gel electrophoresis, with a smear ranging between 500bp and 1000bp considered ideal.

2.2.5.4 Capture Chromatin on Magnetic Beads

To capture the chromatin on magnetic beads, 6.3µg of sheared chromatin was added to 25µl protein G magnetic beads (Active Motif), 10µl ChIP Buffer 1 (Active Motif), 1µl PICt (Active Motif), 3µg of antibody (Table 2.3) and dH_2O to a final volume of 100µl, in a 1.7ml siliconised microcentrifuge tube, per IP. These tubes the samples were incubated at 4°C overnight on a rotating wheel.

2.2.5.5 Elute Chromatin, Reverse Cross-links and Treat with Proteinase K

Following the overnight IP, the samples were briefly centrifuged to collect any liquid remaining in the tubes cap. The samples were then transferred to a clean 1.7ml siliconised microcentrifuge tube to reduce background contamination. The beads were washed 3x with 800µl ChIP Buffer 1 (Active Motif), followed by 3x washes with 800µl ChIP Buffer 2 (Active Motif). Between each wash, the beads were pelleted using magnetic pull down and pulse spins, and the supernatant discarded, before the beads were re-supsended in the appropriate ChIP buffer. Following washing, the beads were re-suspended with 50µl Elution Buffer AM2 (Active Motif) and incubated for 15mins at RT on a rotator wheel, in order to elute the DNA-protein-antibody mix from the beads. To then reverse protein-DNA cross links, each sample was briefly spun and 50µl of the Reverse Cross Link Buffer (Active Motif) was added. The tubes were immediately placed in magnetic stand to allow beads to pellet to sides of tubes. The resulting chromatin containing supernatant was then transferred to fresh tube.

For 'INPUT DNA' 10µl of sheared chromatin was thawed and mixed with 88µl ChIP buffer 2 (Active Motif) and 2µl 5M NaCl. All samples (INPUT and

immunoprecipitated chromatin) were incubated at 65°C for 2.5 hours. The tubes were then allowed to return to RT before 2µl of Proteinase K (Active Motif) was added, and then incubated for 1 hour at 37°C. Samples were again left to return to RT before 2µl Proteinase K Stop Solution (Active Motif) was added. DNA was then used for PCR analysis or stored at -20 °C.

2.2.5.6 PCR screening

To screen immunoprecipitated DNA for protein binding to particular DNA fragments, PCR was employed as described in section 2.2.1.2. The standard PCR protocol using GoTaq polymerase was utilised. Changes to the standard PCR protocol include the use of 10µl immunoprecipitated chromatin sample or 1µl INPUT DNA, per PCR reaction. In general, cycle conditions were as follows: initial denaturation: 95°C for 2mins for 1 cycle; followed by 36 cycles of denaturation: 95°C for 30 seconds, annealing 60-62°C for 30 seconds and elongation: 72°C for 1min; and a final elongation step of 72°C for 2mins.

Gene	Forward (5' – '3)	Reverse (5' – 3')	Spanning
NKB	CTCTAAGCCCCAGCAGTGTCA	CATCCAGCATTCTCCCACTT	-247 to +181
PPTA	GCTGGGATAAATACCGCAAG	CCAACCCACACCTTCAAACCA	-13 to +313
BDNF	GAGATTTTTAAGCCTTTTCCTC	CTTGCCAAGAGTCTATTCC	+22 to +345
Scg10	GCATCCTATCAGTCAGCAA	GGTATGAAACCCTGGCAAGT	-1667 to -1406
L1CAM	GCCCCTCTTTATCTTCTACCTCC	CTGTCCCTGGTGCTGAAATC	+232 to +439
CART	CAGCAACGACGAGTTTCAGA	CACGGCAGAGTAGATGTCCA	+104 to +259

Table 2.4. PCR primers used in ChIP, to amplify region containing known or putative NRSEs. All primers are against human genomic DNA.

2.2.6 Statistics

Results are presented as averages ± standard error of the mean (standard deviation/ Square route of n number) where appropriate. Comparisons were made as appropriate, such as treated Vs control, using the unpaired one-tailed t-test in Microsoft Excel. Significant differences are indicated using: * = $p<0.05$; ** = $p<0.01$ and *** = $p<0.001$.

CHAPTER 3: Regulation of Human NKB (TAC3) gene and its promoter by the transcription factors NRSF and USF

3.1 Introduction

NKB is a member of the tachykinin family and is encoded by the TAC3 gene in humans. NKB, similar to the TAC1 encoded SP, is pro-convulsant in rodent epilepsy models, with elevated NKB immunoreactivity and mRNA levels observed in the rat hippocampus following KA administration (Sperk *et al*., 1990; Marksteiner *et al*., 1992; Wasterlain *et al*., 2002; Chen *et al*., 2008). Furthermore, NKB peptide agonist administration has been shown to exacerbate KA induced neuronal death in mice (Chen *et al*., 2008). In addition to its role in seizure, aberrations of the NKB pathway have been implicated in a number of other defects, including pre-eclampsia (Page *et al*., 2006) and in lung pathophysiology (Pinto *et al*., 2004). Consequently, a greater understanding of NKB regulation would be of importance to a number of fields.

Candidate transcriptional regulators include the TFs NRSF and its truncated isoform sNRSF. Both the rodent NRSF and its truncated isoform (rREST4) are found to be dynamically regulated during models of SSSE (Palm *et al*., 1998), and are both up-regulated following KA stimulation in rodent hippocampal cultures (Spencer *et al.,* 2006), thus matching the observed increase in NKB mRNA following similar treatments (Sperk *et al*., 1990; Marksteiner *et al*., 1992; Wasterlain *et al*., 2002). These zinc finger protein isoforms bind to a 21bp consensus sequence termed the NRSE (Schoenherr & Anderson,1995; Chong *et al.,* 1995). Through binding to target NRSEs, NRSF isoforms

can control temporal and spatial plasticity of neuronal genes in a number of pathways, such as neurogenesis (Ballas *et al.,* 2005; Arora *et al*., 2007). Our group has previously shown that NRSF isoforms regulate the pro-convulsant tachykinin gene TAC1 (Quinn *et al.,* 2002; Spencer *et al.,* 2006), and I propose that NRSF may play a similar regulatory role for NKB. In support of this, the rodent NKB encoding gene (TAC2) was recently proposed to be a potential NRSF target based on the presence of a putative NRSE. This NRSE was however identified over 20Kb upstream from the gene (Otto *et al.,* 2007). I have identified an alternative putative NRSE much closer to the TSS, spanning +50 to +71 of the human NKB gene (TAC3) promoter, based on the consensus sequence (T(C/T)AG(A/C)(A/G)CCNN(A/G)G(A/C)(G/C)AG) (Wu & Xie., 2006; Bruce *et al*., 2006). Thus I set out to investigate the potential regulatory role of NRSF isoforms on NKB promoter activity and expression.

In addition to the putative NRSE identified in this study, I have also identified a single bHLH binding motif (the E box) in the human NKB proximal promoter region, spanning +160 to +166, based on the canonical sequence CANNTG (Sirito *et al*., 1992). This therefore suggests a potential role for bHLH factors in NKB regulation. One such member of the bHLH family of TFs is USF. USF proteins are ubiquitously expressed throughout the body, with particularly high levels of expression found within the brain (Sirito *et al*., 1994). USF proteins have been shown to regulate a number neuronal genes such as GABA receptors (Steiger *et al*., 2004), BDNF (Tabuchi *et al*., 2002) and KCC2 (Markkanen *et al*., 2008), and our group has previously shown that USF modulates rat TAC1 promoter activity in PC12 cells (Paterson *et al*, 1995) and in cultured DRG neurons (Gerrard *et al*, 2005). Interestingly, USF has been shown to regulate a number

of genes targeted by NRSF, including BDNF (Chen *et al*, 2005, Tabbuchi *et al*, 2002) and the AVP promoter (Coulson *et al*, 1999; Quinn *et al*, 2002; Coulson *et al.,* 2003). Subsequently, I postulate that USF may also play a regulatory role for NKB expression, and set out to explore such a mechanism in this chapter.

3.2 Aims

- Test the activity of the human NKB proximal promoter region in cultured human neuroblastoma cells.
- To explore if the NRSF isoforms and the USF proteins regulate the NKB promoter and endogenous NKB gene expression
- To test if this can be modulated using clinically prescribed ACDs.
- To explore potential binding of NRSF and USF to the NKB proximal promoter region via ChIP.
- To explore changes in NKB gene expression following ACD treatment.

3.3 Methods

3.3.1 PCR amplification of NKB promoter and generation of pNKB reporter gene constructs.

The NKB proximal promoter regions spanning -757 to +181 (pNKB-757) and -289 to +181 (pNKB-289) were amplified by PCR as described in methods section 2.2.1.2, using the primers given in Table 2.2.2 and PCR reaction conditions given in Section 2.2.1.6. The amplified NKB promoter regions were cloned into the upstream of the *Firefly* luciferase reporter gene in the promoter-less pGL3basic (pGL3b) vector as described in section 2.2.1.6. The full length human NRSF expression construct (REEX1) used in co-transfection experiments was obtained as described in Chong *et al*., 1995. A truncated expression construct containing 2kb of the human NRSF sequence, analogous to sNRSF (HZ4), was a kind gift from Dr D.J.Anderson. Constructs expressing full length USF1 (pN3) and USF2 (pN4), were a kind gift from Dr.M.Sawadogo. For full descriptions of reporter gene constructs and expression constructs used in transient transfection experiments please see Table 2.1.

3.3.2 Cell culture, treatment, transfections and luciferase assays

Human SK-N-AS cells and SH-SY5Y cells were cultured as outlined in methods section 2.2.2.1.1 and 2.2.2.1.2, respectively, treated as described in section 2.2.2.2 and transfected as described in methods section 2.2.4.2. Cells were then harvested and assayed using the Dual Luciferase Reporter Assay System as outlined in methods section 2.2.6

3.3.3 mRNA expression analysis

The expression of NKB, together with the TFs NRSF, sNRSF, USF1 and USF2, and the house keeping gene Pol-II, was investigated using RT-PCR as described in methods section 2.2.3.4, using the PCR primers set out in Table 2.2.1. Thermal cycle conditions for all expect NKB, were as follows: initial denaturation: 95^{o}C for 2mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds, annealing 62^{o}C for 30 seconds and elongation: 72^{o}C for 1min; and a final elongation step of 72^{o}C for 2mins. For NKB the annealing temperature was adjusted to 64^{o}C, with all other conditions remaining the same.

3.3.4 Transcription factor binding study

To investigate potential TF binding interactions with the NKB proximal promoter region encompassing the putative NRSE and +160 E Box (see Fig 3.1), ChIP was employed as outlined in method section 2.2.5, using the antibodies given in Table 2.3 and the PCR primers given in Table 2.4.

3.4 Results

3.4.1 Identification of a putative NRSE and E box site within the NKB proximal promoter

I have identified a putative NRSE spanning +50 to +71 of the human NKB (TAC3) gene (Figure 3.1a), based on the consensus sequence (T(C/T)AG(A/C)(A/G)CCNN(A/G)G(A/C)(G/C)AG) (Wu & Xie., 2006), incorporating the core sequence CAGCACC (Bruce *et al*., 2006). A sequence comparison between this putative NKB NRSE and that of three other well characterised NRSEs in the NRSF target genes SCN2a, SCG10 and Synapsin, revealed a high degree of homology (Figure 3.1b). Furthermore, the putative NKB NRSE sequence exhibits high homology with the characterised NRSEs of the neuropeptides TAC1 (rat) and AVP (human) (Coulson *et al*., 1999; Quinn *et al*., 2002), especially in the 5' of the consensus (Figure 3.1c).

In addition, I have identified a putative E Box motif spanning +160 to +166 (Figure 3.1a), based on the canonical sequence (CANNTG) (Sirito *et al*., 1992), which USF1 and USF2 are known to bind to with high affinity (Rada-Iglesias *et al*, 2008).

3.4.2 Expression of NKB in neuroblastoma cell lines SK-N-AS and SH-SY5Y

RT-PCR revealed that the human neuroblastoma cell line SK-N-AS expressed NKB, together with the TFs NRSF, USF1 and USF2 (Figure 3.2a), but not the truncated sNRSF isoform. This cell line was chosen to investigate the activity of the NKB proximal promoter, via luciferase reporter gene assays, and to explore modulation of endogenous NKB expression. At a later date, RT-PCR revealed the presence of NKB in

another human neuroblastoma cell line, SH-SY5Y, together with NRSF, sNRSF, USF1 and USF2 (Figure 3.2b). This second cell line was used in the ChIP assays.

3.4.3 NKB proximal promoter supports luciferase reporter gene activity

The neuroblastoma cell line SK-N-AS was used to test the ability of the NKB proximal promoter to support reporter gene expression, due to the endogenous expression of NKB (Figure 3.2a). I cloned two fragments containing the NKB proximal promoter, spanning either -757 to +181 (pNKB-757) or -289 to +181 (pNKB-289) (highlighted in Figure 3.1a), into a promoter-less luciferase reported gene plasmid, pGL3 basic (pGL3b). Both NKB proximal promoter fragments drove significantly elevated levels of luciferase activity compared to the pGL3 basic backbone, with pNKB-757 supporting a 20x fold increase ($P = < 0.001$) and pNKB-289 driving a 35x fold increase in luciferase activity ($P = < 0.001$) (n=9) (Figure 3.3). Interestingly, the shorter NKB construct (pNKB-289) drove significantly more luciferase activity compared to the longer construct (pNKB-757), suggesting the presence of a repressor element within the fragment spanning -757 to -289 ($P = < 0.001$) (n=9) (Figure 3.3).

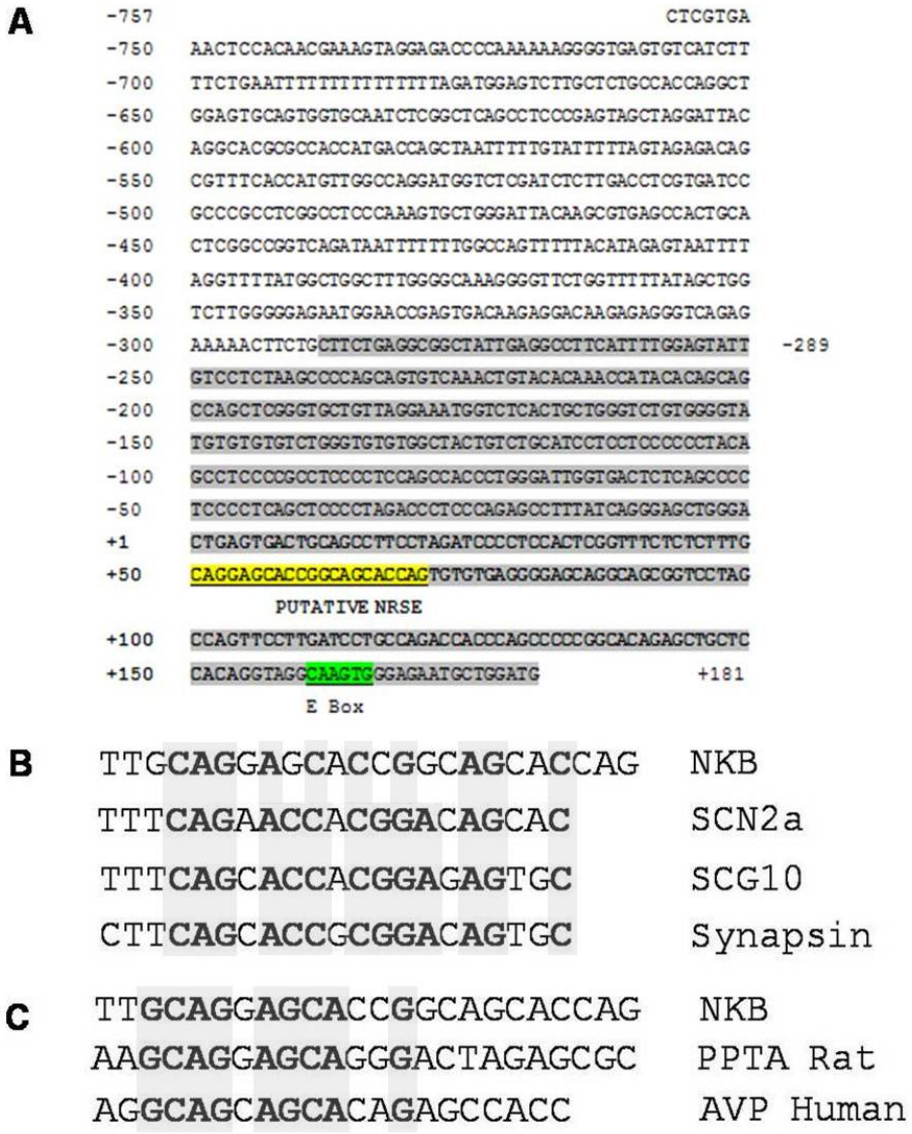

Figure 3.1. (A) The human NKB proximal promoter region spanning -757 to + 181, which was cloned into the pNKB-757 reporter gene construct. Highlighted in grey is the region spanning -289 to +181, cloned into the pNKB-289 reporter gene construct. The putative NRSE spanning +50 to +71 is highlighted in yellow, and the putative E Box spanning +160 to +166 is highlighted in green. **(B)** Sequence comparison of the putative NKB NRSE against known NRSEs within the genes SCN2a, SCG10 and Synapsin. **(C)** Comparison of putative NKB NRSE with NRSEs within t neuropeptide genes TAC1 and AVP.

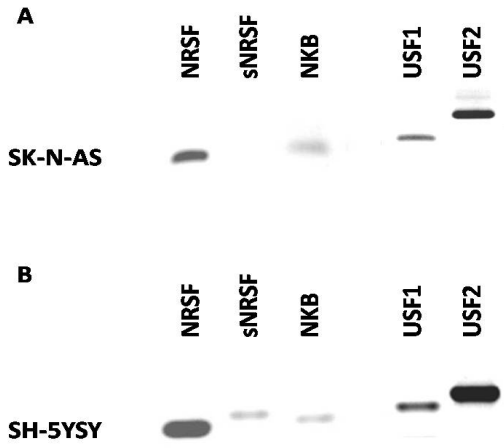

Figure 3.2. Expression profile of the human cell lines SK-N-AS and SH-SY5Y. **(A)** RT-PCR was employed to detect the presence of NRSF, NKB, USF1 and USF2 in human SK-N-AS cells. **(B)** The human SH-SY5Y also expressed NRSF, NKB, USF1 and USF2, as well as the truncated sNFSF isoform.

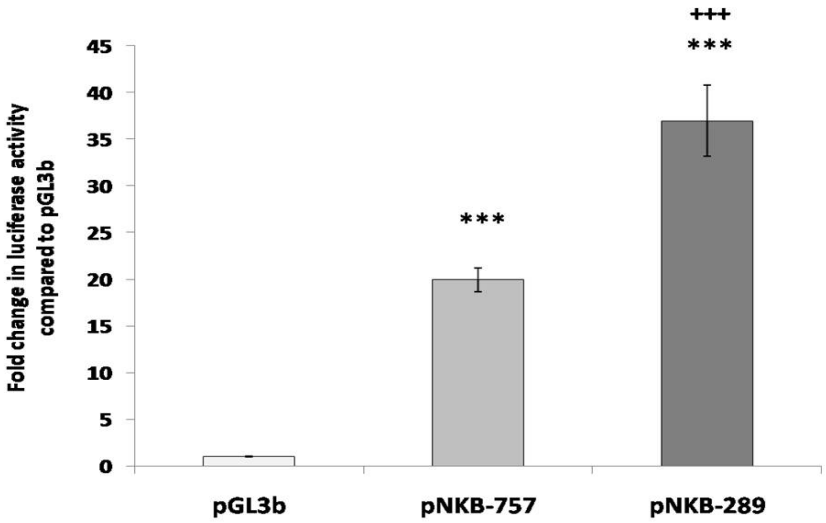

Figure 3.3. Luciferase reporter gene activity driven by the NKB proximal promoter. Both the shorter (-289 to +181) (pNKB-289) and the long (-757 to +181) (pNKB-757) NKB proximal promoters drove high levels of luciferase reporter gene activity when compared to the promoter-less backbone vector pGL3b. pNKB-289 was found to enhance activity to an even greater extent than pNKB-757. Human SK-N-AS cells were transfected with either pNKB-289 or pNKB-757 reporter gene constructs or pGL3b promoter-less backbone vector. Luciferase activity was measured and mean fold changes compared to pGL3b are given. Statistics were performed using *T*-test, with *** = $P < 0.001$ (compared to pGL3b) & +++ = $P < 0.001$ (pNKB-289 Vs. pNKB-757). S.E. are given as Y-axis error bars (n=3, performed in triplicate).

3.4.4 NRSF isoforms activate the NKB promoter and induces its expression

The presence of a putative NRSE within the NKB promoter suggested a role for the TF NRSF in the regulation of the human NKB gene. To address this, I co-transfected human neuroblastoma SK-N-AS cells, with the NKB reporter gene constructs, in combination with plasmids over-expressing either full-length NRSF (REEX1) or a truncated version analogous to the truncated NRSF isoform, sNRSF (HZ4). The HZ4 construct has been used by many groups including our own, to mimic the properties of sNRSF (Roopra *et al*., 2000; Garriga-Canut *et al*., 2006; Spencer *et al*., 2006).

Luciferase activity was measured and over-expression of both REEX1 and HZ4, resulted in a significant increase in the activity of both the long pNKB-757 and the short pNKB-289 reporter gene constructs, compared to controls (reporter gene constructs alone) ($P = < 0.001$) (n=9) (Figure 3.4). The over-expression of both NRSF isoforms resulted in a 3x fold increase in the luciferase activity of the short pNKB-289 construct, compared to pNKB-289 alone control ($P = < 0.001$) (n=9) (Figure 3.4). Furthermore, the over-expression of REEX1 led to a 3.5x fold increase in reporter gene activity of the long pNKB-757 construct, compared to pNKB-757 alone control, which interestingly was found to be statistically higher than the 2x fold increase induced by the over-expression of HZ4 ($P = < 0.001$) (n=9) (Figure 3.4).

To address whether the observed modulation of NKB promoter activity, was manifested in changes in endogenous NKB gene expression, I over-expressed the REEX1 and HZ4 expression constructs for 24hrs or 48hrs and measured expression of the NKB gene by RT-PCR. The over-expression of both NRSF isoforms induced an

increase in the expression of NKB mRNA, which was found to be significant at 48hrs for REEX1 (Figure 3.5a) and 24hrs for HZ4 (Figure 3.5b) ($P = < 0.05$) (n=3).

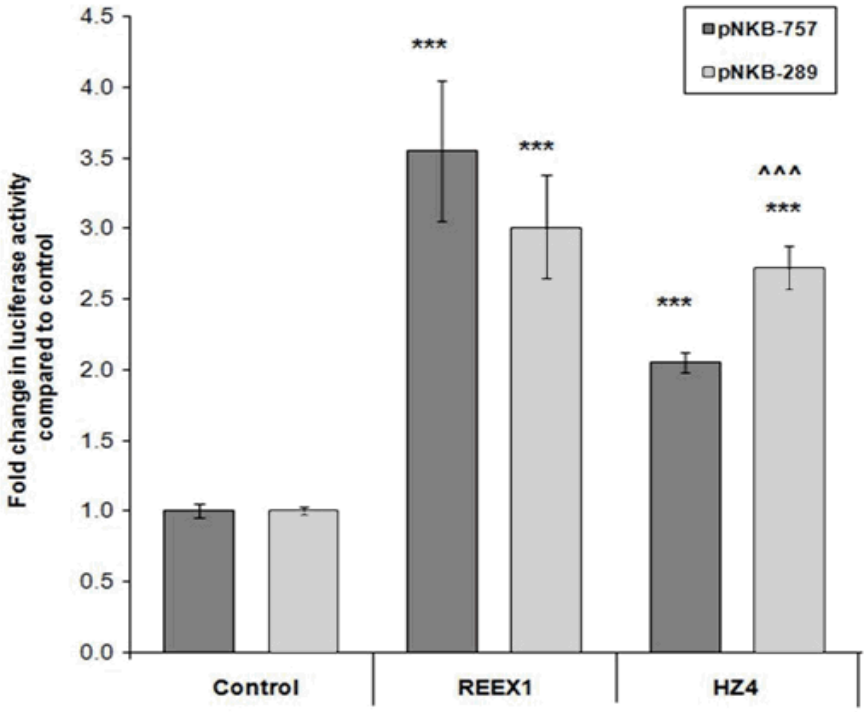

Figure 3.4. Regulation of human NKB promoter by NRSF isoforms in SK-N-AS cells. Over-expression of NRSF (REEX1) and the truncated variant (HZ4) significantly enhances the activity of both pNKB-757 and pNKB-289 reporter gene constructs. Human SK-N-AS cells were transfected with either the short pNKB-289+181 reporter gene construct or the long pNKB-757+181 reporter gene construct, together with either the full length NRSF (REEX1), a truncated version analogous to sNRSF (HZ4) or no expression construct (control). Luciferase activity was measured and mean fold changes compared to controls are given. Statistics were performed using Students T-test, with *** = $P = < 0.001$ (compared to control) and ^^^ = $P = <0.001$ (comparing pNKB-289 vs. pNKB-757). S.E. are given as Y-axis error bars (n=3, performed in triplicate).

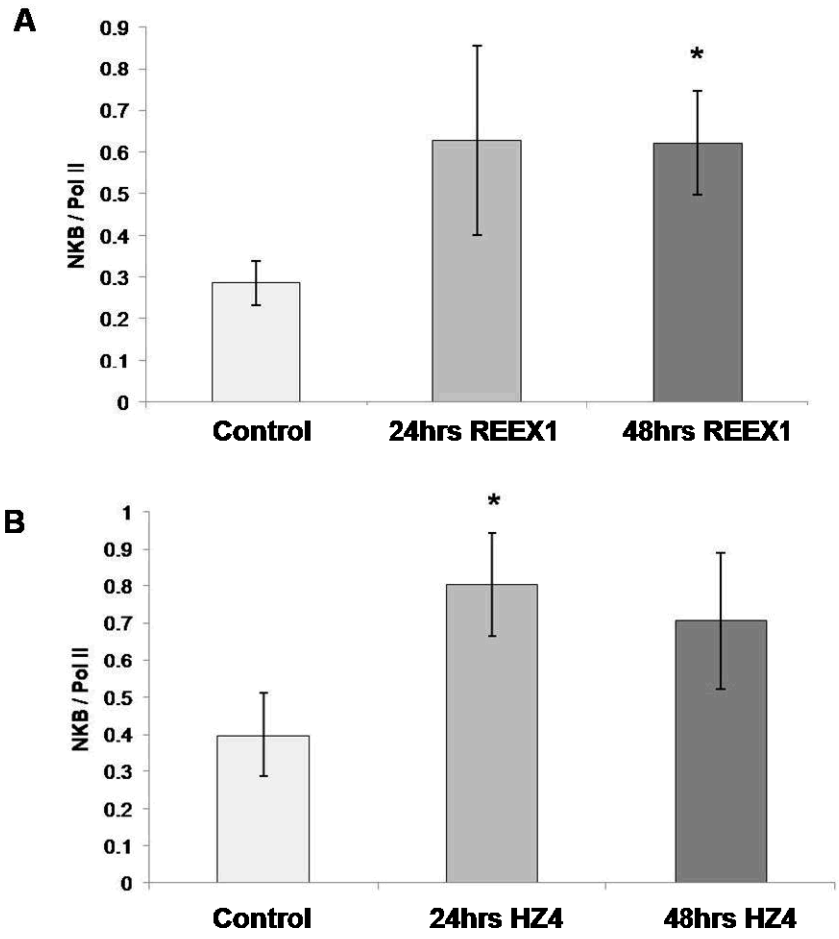

Figure 3.5. Over expression of NRSF (REEX1) and the truncated varient (HZ4) enhances NKB mRNA expression. (A) The over-expression of full length NRSF (via the REEX1 expression construct) and (B) a truncated variant (via the HZ4 expression construct) led to the significant increase in NKB mRNA levels, following 48hrs (REEX1) and 24hrs (HZ4) over-expression. Changes in endogenous NKB mRNA levels following 0hrs (control), 24hrs and 48hrs REEX1 or HZ4 over-expression, where measured via RT-PCR, with band intensities normalized against Pol II, * = $P = < 0.05$. (n=3).

3.4.5 USF1 and USF2 repress the NKB promoter

The presence of a putative E Box motif spanning +160 to +166 within the human NKB promoter suggested a possible role for the TFs USF1 and USF2 in the regulation of the NKB gene. To address this, I co-transfected human neuroblastoma SK-N-AS cells, with the NKB reporter gene constructs, in combination with plasmids over-expressing either full-length USF1 (pN3) or full-length USF2 (pN3). Luciferase activity was measured and the over-expression of both USF1 and USF2 resulted in the significant repression of both the long (pNKB-757) and short (pNKB-289), compared to controls ($P = < 0.001$) (n=9) (Figure 3.6). The over-expression of USF1 resulted in an almost identical 40% reduction in luciferase activity driven by both the long and short NKB constructs. USF2 over-expression resulted in a similar 40% reduction in luciferase activity driven by the longer (pNKB-757) construct, whilst eliciting even greater repression of the short pNKB-289 construct, with a 60% reduction in luciferase activity compared to control (no expression construct) alone ($P = 0.001$) (n=9) (Figure 3.6).

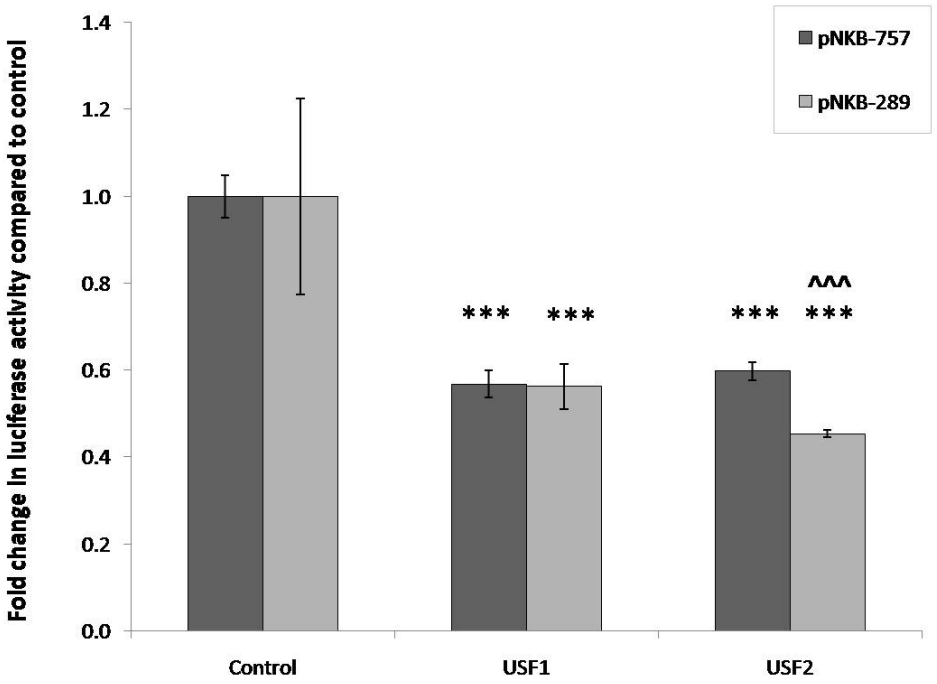

Figure 3.6. Regulation of human NKB promoter by USF proteins in SK-N-AS cells. Over-expression of both USF1 and USF2 significantly represses the activity of both pNKB-757 and pNKB-289 reporter gene constructs. Human SK-N-AS cells were transfected with either the short pNKB-289+181 reporter gene construct or the long pNKB-757+181 reporter gene construct, together with either USF1 (pN3), USF2 (pN4) or no expression construct (control). Luciferase activity was measured and mean fold changes compared to controls are given. Statistics were performed using Students T-test, with *** = *P = < 0.001* (compared to control) and ^^^ = *P = <0.001* (comparing pNKB-289 vs. pNKB-757). S.E. are given as Y-axis error bars (n=3, performed in triplicate).

3.4.6 Transcription factor binding to the NKB promoter revealed by ChIP

To determine if the NRSF isoforms regulate NKB expression directly via binding to the NKB proximal promoter, I analysed binding of both NRSF and the truncated isoform sNRSF to the region encompassing the NKB promoter. Furthermore, as USF1 and USF2 repressed NKB promoter activity, I also analysed binding of both USFs to the same region. I used PCR to identify the region spanning -247 to +181

(which encompassed both the putative NRSE and the E Box motif) in the material enriched by the chromatin ChIP.

In the human SK-N-AS cell line, there was no strong evidence for sNRSF binding to the NKB promoter, as indicated by comparison with the non-specific background (negative) control; IgG band (Figure 3.7a). This was as expected due to the absence of sNRSF in the SK-N-AS cell line, as determined by RT-PCR (Figure 3.2a). In contrast, the full-length isoform did appear to exhibit weak binding to the NKB promoter, when compared against the IgG negative control. In order to investigate sNRSF binding to the NKB promoter and to confirm the weak NRSF binding, I utilised a second human neuroblastoma cell line, SH-SY5Y, which expressed both NRSF and sNRSF isoforms, as well as NKB (Figure 3.2b). In human SH-SY5Y cells, both NRSF and sNRSF exhibited strong binding to the NKB promoter, when compared against the non-specific background control; IgG (Figure 3.7b). Binding of NRSF and sNRSF to the NKB promoter was found to be specific, as neither NRSF nor sNRSF IPs gave rise to an amplification of the control GAPDH PCR product.

In addition, USF binding to the NKB promoter region was also observed in both SK-N-AS and SH-SY5Y cell lines. Interestingly, in the SK-N-AS cell line, USF1 was found to bind to the NKB promoter (based on stronger PCR band intensities compared to the IgG negative control), but USF2 was absent (Figure 3.7a). In direct contrast, USF2 was found to bind the NKB promoter in SH-SY5Y cells, but USF1 was absent (Figure 3.7b).

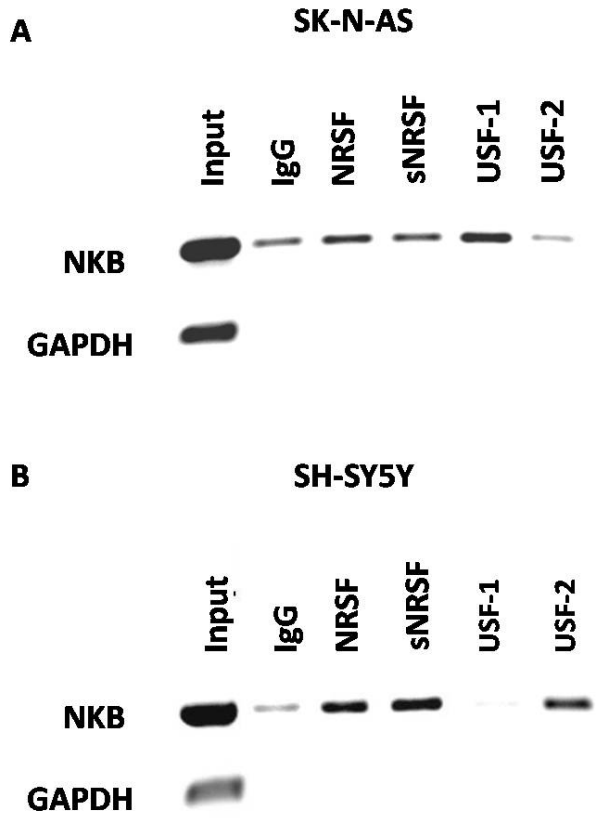

Figure 3.7. NRSF isoform and USF binding to the NKB proximal promoter. (A) ChIP analysis revealed that only USF1 exhibited strong binding to the NKB promoter in untreated human SK-N-AS cells, compared to IgG negative control. NRSF displays weak, if any binding to the NKB promoter, with no sNRSF or USF2 bindng obsereved. **(B)** In contrast, both NRSF and sNRSF were found to bind to the NKB promoter in untreated human SH-SY5Y cells compared to IgG negative control, as was USF2, but no USF1 binding was observed. Binding is shown to be specific to the NKB proximal promoter, as no binding is observed in the GAPDH PCR negative controls (n=1).

3.4.7 The anticonvulsant CBZ impairs both NRSF isoform and USF regulation of the NKB promoter and represses NKB expression

The data presented here indicates that both NRSF isoforms and the USF TFs are capable of regulating the pro-convulsant neuropeptide NKB promoter. I wished to explore whether ACD treatment could modulate NRSF and USF-mediated regulation of

NKB, as a potential novel mechanism of action for ACDs. Treatment of human SK-N-AS cells with 50µg/ml CBZ, for 24hrs following transfection, resulted in no significant change in luciferase reporter gene activity, driven by neither the long (pNKB-757) nor the short (pNKB-289) NKB promoter (Figure 3.8). 24hrs 50µg/ml CBZ did however cause a marked impairment of both NRSF isoform and USF regulation of the NKB reporter gene constructs. The observed activation of both the long (pNKB-757) and short (pNKB-289) NKB reporter gene constructs following over-expression of either NRSF (REEX1) or the truncated construct (HZ4), was significantly reduced following 24hrs 50µg/ml CBZ ($P = < 0.001$) (n=9) (Figure 3.8a and Figure 3.8b, respectively). The observed CBZ induced impairment, was greatest for the shorter pNKB-289 construct, with CBZ inducing a 3x fold reduction in luciferase activity following either REEX1 or HZ4 over-expression, returning levels close to untreated control levels ($P = < 0.001$) (n=9) (Figure 3.8b).

Treatment of SK-N-AS cells with CBZ also resulted in a significant impairment of USF regulation of the NKB reporter gene constructs. The observed repression of luciferase activity following both USF1 and USF2 over-expression was found to be abolished following 24hrs 50µg/ml CBZ treatment. USF1 and USF2 repression of the long pNKB-757 reporter gene construct was significantly modulated by CBZ treatment, with luciferase activity returning back to untreated control levels for USF2 and greater still, leading to enhanced luciferase activity with regards to USF1 (Figure 3.9a) ($P = <0.001$) (n=3). The luciferase activity driven by the shorter pNKB-289 construct was also returned to untreated control levels, following CBZ treatment (Figure 3.9b) ($P = <0.001$) (n=3).

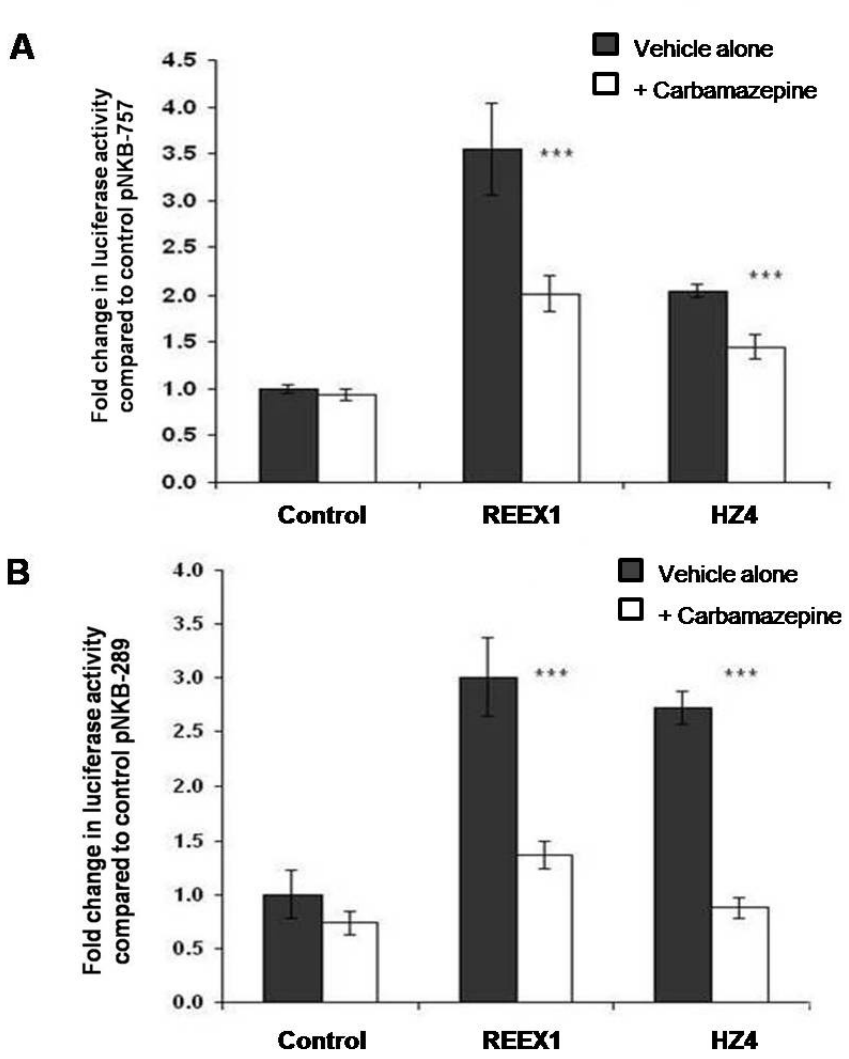

Figure 3.8. Effect of anticonvulsant drug treatment on the regulation of NKB promoter by NRSF isoforms. Anticonvulsant drug treatment of human SK-N-AS neuroblastoma cells resulted in a significant decrease in response of both the (A) pNKB-757 and (B) pNKB-289 reporter gene construct to the over-expression of both full length NRSF (REEX1) and truncated isoform sNRSF (HZ4). Human SK-N-AS cells were transfected with either the pNKB-757 or the pNKB-289 reporter gene construct alone (control) or co-transfected with either the full length NRSF (REEX1) or the truncated isoform sNRSF(HZ4) expression constructs. Cells were either treated with vehicle alone (black bars) or treated with 50µg/ml CBZ for 24hrs following transfection (white bars). Luciferase activity was measured and mean fold changes compared to controls are given. Statistics were performed using Students *T*-test comparing treated vs. untreated, with *** = $P = < 0.001$. S.E. are given as Y-axis error bars (n=3, performed in triplicate).

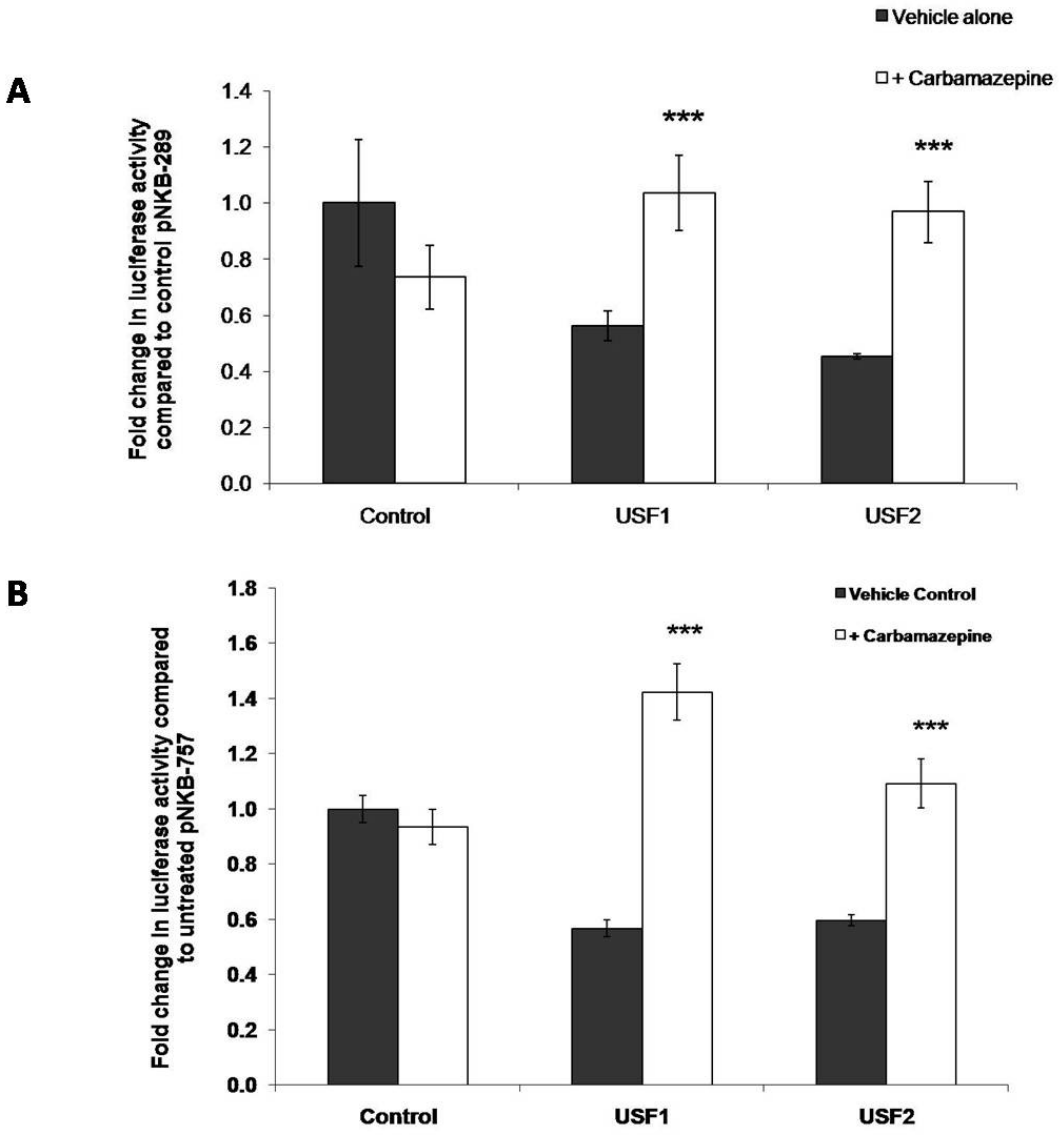

Figure 3.9. Effect of anticonvulsant drug treatment on the regulation of NKB promoter by USF. Anticonvulsant drug treatment of human SK-N-AS neuroblastoma cells resulted in a significant increase in response of both the (A) pNKB-757 and (B) pNKB-289 reporter gene construct to the over-expression of both USF1 and USF2. Human SK-N-AS cells were transfected with either the pNKB-757 or the pNKB-289 reporter gene construct alone (control) or co-transfected with either USF1 (pN3) or USF2 (pN4) expression constructs. Cells were either treated with vehicle alone (black bars) or treated with 50µg/ml CBZ for 24hrs following transfection (white bars). Luciferase activity was measured and mean fold changes compared to controls are given. Statistics were performed using Students *T*-test comparing treated vs. untreated, with *** = $P = < 0.001$. S.E. are given as Y-axis error bars (n=3, performed in triplicate).

To determine the impact of CBZ induced impairment of NRSF and USF regulation of the NKB promoter, on NKB expression, RT-PCR was employed. Expression of the endogenous NKB gene was also found to be significantly repressed following 24hrs 10µg/ml CBZ treatment when compared to the housekeeping gene RNA polymerase II, ($P = < 0.05$) (n=3) (Figure 3.10), but not by 24hrs 50µg/ml CBZ. This data suggests that the anticonvulsant CBZ may function by repressing NKB expression, perhaps though the inhibition of NRSF and sNRSF regulation of the NKB promoter, indicating a novel mechanism of action for this anticonvulsant drug.

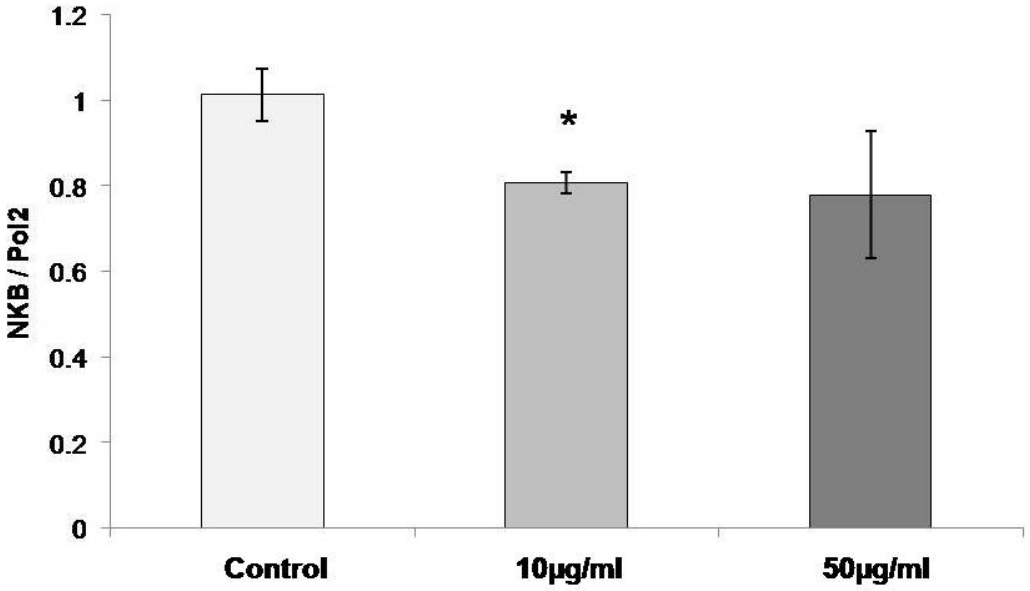

Figure 3.10. CBZ treatment modulates NKB mRNA expression. SK-N-AS cells were treated with 10µg/ml, 50µg/ml or vehicle alone (control) for 24hrs, and immediately processed for RNA extraction. Changes in endogenous NKB mRNA levels were measure via RT-PCR, with band intensities normalised against RNA polymerase II (Pol II), with averages given. Statistics were performed using Students *T*-test, with * = $P = < 0.05$. S.E. are given as Y-axis error bars (n=3).

3.5 Discussion

NKB, like the related tachykinin SP, has been shown to be pro-convulsant in rodent epilepsy models, with elevated NKB immunoreactivity and mRNA levels observed in the rodent hippocampus following KA treatment (Sperk *et al*., 1990; Marksteiner *et al*., 1992; Wasterlain *et al*., 2002; Chen *et al*., 2007). We, and others, have shown that NRSF isoforms are one of the initial targets increased in rodent models of SSSE (Palm *et al*., 1998; Spencer *et al*., 2006), and our identification of a putative NRSE within the NKB proximal promoter suggested a potential regulatory role of NRSF isoforms in governing NKB expression.

The data presented in this chapter indicates that NRSF isoforms are indeed capable of modulating NKB expression, with over-expression of both full length NRSF (through the REEX1 expression construct), and a truncated version (through the HZ4 construct), resulting in the elevation of NKB mRNA in human neuroblastoma cells (Figure 3.5). Furthermore, NRSF isoforms positively regulated the NKB promoter, with both REEX1 and HZ4 over-expression resulting in significantly elevated luciferase activity of reporter gene constructs driven by both the long (pNKB-757) and short (pNKB-289) NKB promoter (Figure 3.4). Preliminary ChIP assays indicated that NRSF isoforms could bind to the region spanning the NKB promoter, most prominently observed in the human SH-SY5Y neuroblastoma cell line (Figure 3.7), which suggests that NRSF regulation of NKB may be through a direct mechanism. However, it should be stated that I have yet to prove that the NRSF isoforms act via, or binds directly to, the putative NRSE, merely that NRSF isoforms bind to the region encompassing that NRSE. To this aim, a cloning strategy was undertaken to remove the putative NRSE

from the NKB reporter gene constructs, and the resulting mutant construct would have been subjected to similar NRSF over-expression studies, as described in this chapter. Unfortunately, unavoidable cloning issues have delayed the generation of this mutant NKB construct, preventing this experiment being undertaken in time for this thesis, however work is continuing at present to generate these mutant constructs for future study.

A second set of TFs, the USF family, were also thought to be potential regulators of NKB expression, based on the identification of a putative E box motif (CANNTG), spanning +160 to +166 of the NKB promoter. In addition, USF proteins have been shown to be important in regulating a number of epilepsy associated genes including GABA receptors (Steiger *et al*., 2004) and BDNF (Tabuchi *et al*., 2002), and our group has shown that USFs are key regulators of the related SP encoding gene, TAC1. Thus I set out to explore a potential role of USF1 and USF2 in NKB regulation.

Both USF1 and USF2 were found to repress or silence luciferase activity driven by both the long (pNKB-757) and short (pNKB-289) NKB reporter gene constructs, (Figure 3.6) suggesting a role for USF proteins in NKB promoter regulation. In support of this, as with the NRSF isoforms, preliminary ChIP assays indicated that both USF1 and USF2 could bind to the NKB promoter region, encompassing the putative E box motif (Figure 3.7). This suggests that any USF regulation of NKB may function via direct binding to its promoter. However, as with the NRSF isoforms, I have not proved that neither USF1 nor USF2 binds specifically to the +160 E Box, only that they bind to the region encompassing that E box, and a cloning strategy to mutant the +160 E box within the NKB reporter gene constructs, would be required to demonstrate specific

binding. Furthermore, the affect of USF over-expression on NKB mRNA expression, has yet to be determined, and would be an important future experiment, together with the generation of the E box mutant, to better understand USF regulation of the human NKB gene.

Interestingly, binding of USF1 and USF2 to the NKB promoter region, was found to be cell specific, with USF1 only, bound to the NKB promoter in SK-N-AS cells, and in contract, USF2 only, was found to bind to the same region in SH-SY5Y cells (Figure 3.7). Expression profiling indicated that USF1 and USF2 are expressed in both cell lines (Figure 3.2) and so differential expression of USF proteins between cell lines cannot explain the observed difference in binding. In general, USF1 is found to bind to virtually all E box sites to which USF2 is known to bind (Rada-Iglesias *et al*., 2008), suggesting competition between USF1 and USF2 for binding sites may exist. It is therefore plausible that competition for binding at that locus between USF1 and USF2 prevents the binding of both TFs to the NKB promoter, resulting in only one bound at any one time. It would thus be of interest to repeat the ChIP assay at different time points in both cell lines, to determine whether this differential binding was solely cell specific, or whether temporal plasticity of USF binding at this region was taking place. Alternatively, it has been shown that USF1 and USF2 can bind to different proteins, and thus expression of these proteins may modulate USF binding to the NKB promoter locus, in a cell specific manner. For example, USF1 has been shown to form a complex with the bHLH TF Cha, with the resulting complex able to bind to E box DNA elements (Rodriquez *et al*., 2003). Therefore, it would be of interest to characterise the expression of such bHLH factors in both cell lines, to address the differential binding observed.

Finally, it is also known that many proteins target and bind to E box DNA elements, such as Mitf (Askan & Goding., 1998) and Myc (Fisher *et al*., 1993), and thus may compete with USF1 or USF2 for binding privileges, and thus monitoring both expression and binding of these TFs to the NKB promoter locus, in each cell line, would be of particular interest.

The data presented in this chapter suggests an activator role for NRSF isoforms in NKB regulation, and a repressive regulatory role for USF proteins. Interestingly, this is in contrast to the classical roles of these TFs. NRSF is classically thought of as a repressor, after first being discovered as a repressor of neuronal genes in non-neuronal cells (Schoenherr & Anderson., 1995; Chong *et al*., 1995), and shown to repress a host of genes including SCN2a (Tapia-Ramirez *et al*., 1997), L1CAM (Kallunki *et al*., 1997) and Scg10 (Chen *et al*., 1998). In comparison, USF proteins are in general considered to act as activators, based on the recent findings showing an association between acetylated histone H3(H3ac) localisation and USF binding to target genes' TSS (Rada-Iglesias *et al*., 2008). Furthermore, we and others have shown USF activates a range of genes including TAC1 (Quinn *et al*., 2002), hepatic lipase (Van Deursen *et al*., 2009) and follicle stimulating hormone (FSH) (Hermann *et al*., 2008).

Despite NRSF and USF regulation of the human NKB being in contrast to the classical regulatory roles of these TFs, there are examples of both NRSF and USF performing these contradictory roles in other genes. We have previously shown that both NRSF isoforms activate the related tachykinin gene TAC1 in rodent hippocampus (Spencer *et al.,* 2006), and this regulatory roles appears to be cell specific, with repression and activation of the TAC1 promoter and endogenous expression observed in

a tissue dependent manner (Quinn *et al.,*2002;Spencer *et al.,* 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009). The truncated isoform has also been shown to act as an activator of the TAC1 promoter in epilepsy (Spencer *et al*., 2006) and another neuropeptide; AVP (Coulson *et al*., 2000; Quinn *et al*., 2002).

Interestingly, in all three neuropeptides shown to be activated by NRSF isoforms (TAC1, NKB and AVP), the location of the NRSEs are close to, or at the major TSS, whereas the majority of all other identified NRSF binding sites (Bruce *et al.,* 2006) have NRSEs that are quite distant from TSS. This location may be important in governing NRSF regulation, with a closer proximity of the NRSE to the TSS, perhaps impairing NRSF recruitment of co-repressors, and thus pushing NRSF function from a repressor to an activator. In support of this, a study by Bessis *et al*, revealed that the location of the NRSE can confer whether the NRSE acts as an repressor or an activator. NRSEs located within 50bp of the TATA box of a synthetic promoter, were found to act as activator elements in neuronal cells, whilst those further upstream acted as repressor elements (Bessis *et al*., 1997).

The NKB putative NRSE is located +51 from the TSS and thus is close to the boundary indicated by Bessis *et al*, suggesting that the putative NKB NRSE could act as an activator element, which is consistent with the activator roles of NRSF isoforms observed here. In addition, the other genes shown to be activated by NRSF (TAC1 and AVP) have similar NRSE locations, with the rat TAC1 NRSE located at -21 to +4 (Quinn *et al*., 2002) and the AVP NRSE -2 to +23 (Coulson *et al*., 1999). Thus the close proximity of these NRSEs to the TSS, may confer transcriptional activator roles

Furthermore, when I compared the TAC1, AVP and NKB (putative) NRSEs (Figure 3.1) I found stronger conservation at the left hand side of the NRSE consensus sequence (nucleotides 2-10) (Appendix 5). Recently the classic 21bp canonical NRSE sequence has been shown to be a bipartite sequence, with a left half-site and a right half-site. Interestingly, whilst NRSF can bind to either half-site, individually neither half-site is an effective repressor, with NRSF binding to both half-sites required for repression (Patel *et al*., 2007). The homology of the left hand side of the TAC1, AVP and (putative) NKB NRSEs, matches that of the left-half site, suggesting in all three cases NRSF binding occurs, but is unable to induce repression. This may indicate that a subset of NRSF target genes exist, which share a NRSE consensus sequence exhibiting stronger homology to the NRSE left-half site, and perhaps this subset is regulated differently to those genes with more complete NRSEs.

As highlighted earlier, USF1 and USF2 also play a regulatory role which is in contrast to their typically described activator roles. However, USF mediated suppression of gene expression has been previously documented, with USF1 and USF2 shown to repress the promoter activity of human telomerase reverse transcriptase (hTERT), through direct binding to its target E box (Chang *et al*., 2005). Furthermore, USF have been shown to recruit a host of histone modifying enzymes, including the H3K4-specific methyltransferase SET7/9 and the H3-specific HAT (West *et al*., 2004). Differential recruitment of these histone modifying enzymes could therefore have a impact on USF regulatory function, and it would thus be important to clarify both the expression of these enzymes in our cell lines, and also determine whether or not these enzymes are bound to the NKB +160 E box, through ChIP assays.

Finally, consistent with a model in which NRSF modulated NKB expression is a mechanism that operates during epilepsy, the governed increase in NKB endogenous expression and promoter activity by the NRSF isoforms is inhibited by the action of the anticonvulsant CBZ. 24hrs 50µg/ml CBZ resulted in a reduction in luciferase activity driven by the NKB promoter following REEX1 or HZ4 over-expression (Figure 3.8). This concentration had limited impact on endogenous NKB mRNA expression, however, a lower concentration of 24hrs 10µg/ml, did lead to a significant reduction in NKB mRNA expression (Figure 3.10). This may in part, be due to the fact that NKB promoter repression induced by USF1 and USF2 over-expression is quenched following 24hrs 50µg/ml CBZ (Figure 3.9), suggesting this concentration of CBZ not only disrupts NRSF activation of NKB, but also USF-mediated repression. The disruption of both regulatory mechanisms by 24hrs 50µg/ml CBZ may result in neither activation nor repression of endogenous NKB occurring, and thus no change in NKB expression. It would be of interest to explore the impact of 24hrs 10µg/ml CBZ on NRSF and USF-mediated regulation of the NKB promoter, to better understand the repression of endogenous NKB expression following such treatment.

CHAPTER 4: Differential regulation of the rat TAC1 promoter by NRSF and sNRSF, in cooperation with USF family members

4.1 Introduction

In the previous chapter, the regulation of the human NKB gene (TAC3) was in part, found to be coordinated by two distinct TF families, the NRSF isoforms and the USF proteins. In a number of instances, both NRSF and USF proteins have been shown to regulate the same gene. Examples include the regulation of AVP (in which USF proteins have been shown to activate the AVP promoter, whilst NRSF over-expression silences the AVP promoter (Coulson *et al*., 1999; Coulson *et al*., 2003)), BDNF (with USF considered as a BDNF promoter activator (Tabuchi *et al*., 2002a), whilst NRSF represses the BDNF promoter, and the truncated isofrom activates the BDNF promoter (Tabuchi *et al*., 2002b)) and now NKB. Both TF families have been implicated in cartain diseases such as epilepsy (Palm *et al*., 1998; Sirito *et al*., 1998), SCLC (Coulson *et al*., 1999; Coulson *et al.,* 2003) and breast cancer (Ismail *et al*., 1999; Patel *et al*., 2005; Reddy *et al*., 2009).

In addition to NKB, AVP and BDNF, another NKB related tachykinin gene, TAC1, has also been shown by our own group, and others, to be regulated by both NRSF and USF proteins. The TAC1 encoded gene SP, is implicated in a wide range of neurological disorders including epilepsy (Liu *et al*., 1999; Wasterlain *et al*., 2000), Parkinson's disease (Barker., 1991; Chen *et al*., 2004), HD (Richfield *et al*., 2002), pain (Duggan *et al*., 1987; Duggan *et al*., 1988) and cognitive and psychiatric disorders (Kramer *et al*., 1998; Maubach *et al*., 1999).

The NRSF binding sequence, the NRSE, has been identified in both the rat (Quinn *et al*., 2002) and the human (Greco *et al.*, 2007) TAC1 proximal promoter regions (near the TSS), and has been shown to regulate TAC1 promoter activity and expression. This regulatory role was found to be cell specific, with repression and activation of the TAC1 promoter and endogenous expression observed in a tissue dependent manner (Quinn *et al.,*2002;Spencer *et al.,* 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009). Similarly, USF has also been shown to modulate rat TAC1 promoter activity in PC12 cells (Paterson *et al*., 1995). This modulation is thought to occur through USF recognising and binding to a canonical E box at -60 (CACGTG). Mutation of this E box has been shown to abolish the ability of a reporter gene construct spanning -865 to +92 of the rat TAC1 promoter, to support reporter gene expression in PC12 cells (Paterson *et al*., 1995). Additionally, this -60 E box has been shown to be important in coordinating the response of the TAC1 promoter to NGF stimulation (Gerrard *et al*., 2005).

Furthermore, both USF and NRSF are known to recruit chromatin remodelling enzymes, to bring about changes in chromatin structure such as histone methylation and acetylation. USF has been shown to recruit enzymes which can acetylate histones (PCAF) and methylate histone H3K4 (SET7/92) (West *et al.,* 2004). Whereas NRSF has been shown to recruit a multitude of enzymes and cofactors including mSin3, HDAC1 and HDAC2, CoREST, CtBP and G9a (reviewed in Ooi & Wood, 2007).

Due to NRSF and USF having been shown to regulate a number of shared target genes (NKB, AVP, BDNF and TAC1), I was interested in exploring the possibility of an inherent mechanistic relationship existing between NRSF and USF in the regulation of

neuropeptides. I have focused on the rat TAC1 promoter based on the presence of a single NRSE, multiple USF binding sites, and the availability of reporter gene constructs with mutations in the key -60 E box site. The rat TAC1 promoter shares a good degree of homology with the human TAC1 promoter region, with the E box motifs located at -60, -170 and -308 in the rat TAC1 promoter, perfectly conserved in the human (Appendix 3). Furthermore, the characterised rat TAC1 NRSE is also well conserved in the human (65% homology), however this is not the same NRSE as that proposed and characterised by the Rameshwar group (Greco *et al*., 2007), and thus will be referred to as a putative human TAC1 NRSE.

4.2 Aims

- Test the activity of the rat TAC1 reporter gene construct spanning -865 to +92 (-856 TAC), together with the mutant construct, containing a mutation at the -60 E box (-60 TAC), in the TAC1 expressing SK-N-AS human neuroblastoma cell line.
- To determine the impact of NRSF isoform and USF protein over-expression on both reporter gene constructs.
- To explore the impact of over-expressing NRSF isoforms and USF proteins in combination with each other.
- To determine TF binding to the human TAC1 promoter in human neuroblastoma cell lines (SK-N-AS and SH-SY5Y) using ChIP.

4.3 Methods

4.3.1 Cell culture, transfections and luciferase assays

Human SK-N-AS cells were employed for reporter gene assays based on the observed endogenous expression of TAC1 as revealed by RT-PCR (Figure 4.2). In addition, the sNRSF-expressing human SH-SY5Y neuroblastoma cell line was employed in ChIP assays to explore sNRSF binding to the human TAC1 promoter. SK-N-AS and SH-SY5Y cells were cultured as outlined in methods section 2.2.2.1.1 and 2.2.2.1.2, respectively, in sterile 24-well plates (reporter gene assays) or T75 flasks (ChIP assay). SK-N-AS cells were transfected as described in methods section 2.2.4.2, and harvested and assayed for luciferase reporter gene assays as outlined in methods section 2.2.6. Cells were harvested and processed for ChIP assays as described in section 2.2.5.

To explore the regulation of the TAC1 promoter by the TFs NRSF and USF, both alone and in combination, I opted to utilise the rat TAC1 promoter reporter gene construct -865 TAC1, which contains the rat TAC1 promoter region spanning -865 to +92. This promoter region contains both an NRSF binding site (the NRSE), and multiple USF binding motifs (E boxes), as shown in Figure 4.1. Furthermore, a second reporter gene construct containing a disruptive 10bp insertion in the -60 E box (-60 TAC) (Paterson *et al*., 1995) was utilised. This -60 E box has previously been shown to be important in USF regulation of the rat TAC1 promoter (Paterson *et al*., 1995; Gerrard *et al*., 2005), and I was interested in exploring a role of this E box in NRSF regulation of

the rat TAC1 promoter. Over-expression constructs employed are described in section 2.1.1.

4.3.2 mRNA expression analysis

The expression of TAC1, together with the TFs NRSF, sNRSF, USF1 and USF2 was investigated using PCR as described in methods section 2.2.3.4, using the PCR primers set out in Table 2.2.1. Thermal cycling conditions for all were as follows: initial denaturation: 95^{o}C for 2mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds, annealing 62^{o}C for 30 seconds and elongation: 72^{o}C for 1min; and a final elongation step of 72^{O}C for 2mins.

4.3.3 Transcription factor binding study

To investigate potential TF binding interactions with the human TAC1 proximal promoter region encompassing the characterised NRSE (Greco *et al*., 2007), the putative human TAC1 NRSE (based on homology to the rat TAC1 NRSE) (Appendix 2 & 3) and multiple E Box motifs, ChIP was employed as outlined in method section 2.2.5, using the antibodies given in Table 2.3 and the PCR primers given in Table 2.4.

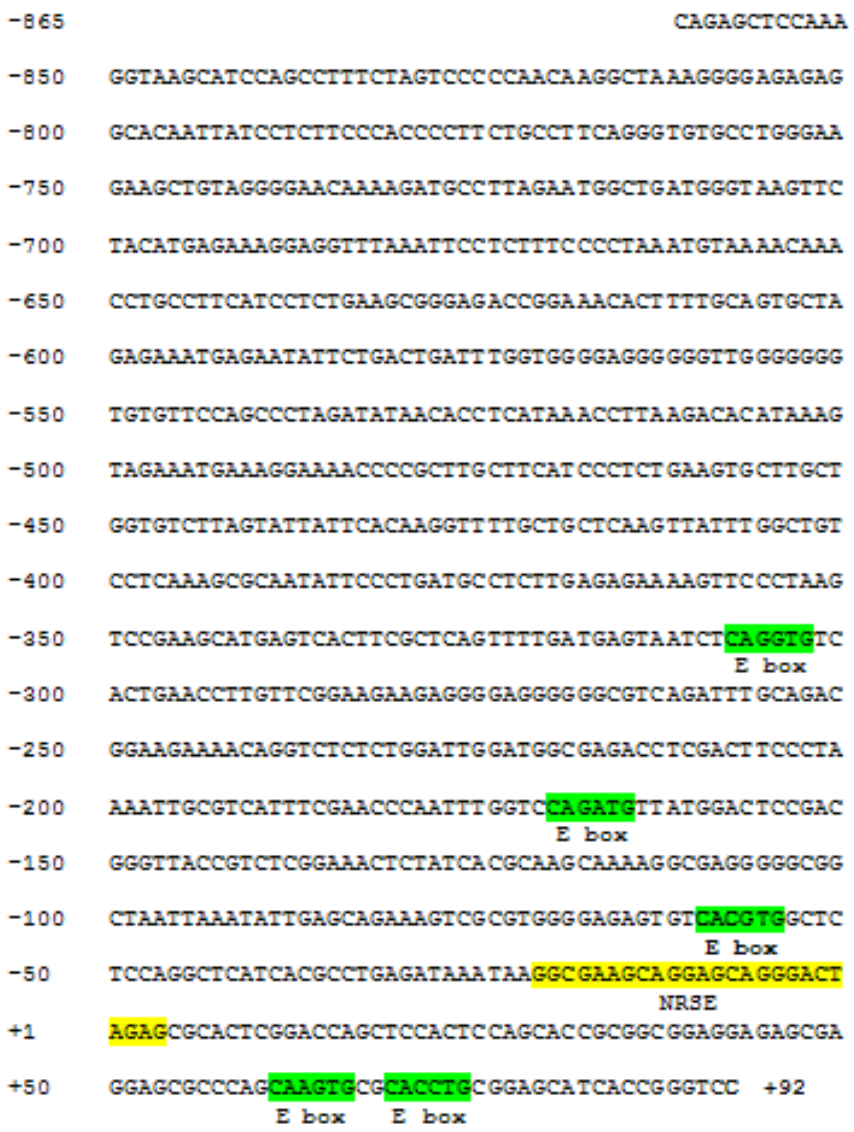

Figure 4.1 Sequence of the rat TAC1 promoter region spanning -865 to +92. The rat TAC1 promoter region (utilised in the -865 TAC1 reporter gene construct) contains multiple bHLH binding motifs (E box) highlighted in green, and a single NRSE, highlighted in yellow.

4.4 Results

4.4.1 Expression of TAC1, NRSF, USF1 and USF2 in human SK-N-AS neuroblastoma cells

The regulation of the TAC1 promoter will be determined by the TFs present in the cell. Consequently, it was important to use an appropriate cell line which expressed TAC1 endogenously. I was however limited in the number of clonal cell lines that express the endogenous TAC1 gene (Quinn *et al*., 2000). By screening a number of clonal cell lines in our lab by RT-PCR, I found that the human neuroblastoma cell line SK-N-AS, expressed TAC1 endogenously (Figure 4.2). I therefore used this cell line to investigate the effect of over-expression of the NRSF and USF TFs, on the activity of the TAC1 promoter. Furthermore, RT-PCR showed the presence of NRSF, USF1 and USF2 in SK-N-AS cells, but the truncated NRSF isoform, sNRSF, was non-detectable.

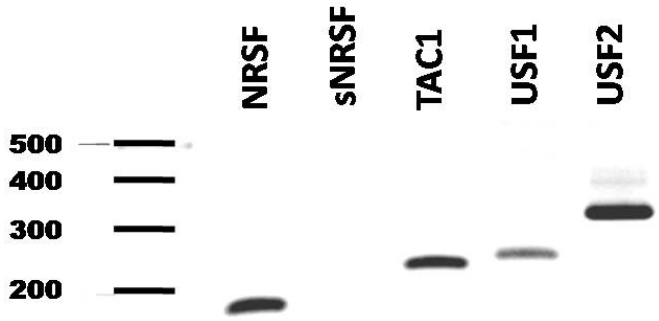

Figure 4.2 Expression profile of TFs in human SK-N-AS neuroblastoma cells. RT-PCR revealed that human SK-N-AS cells express full length NRSF, TAC1, USF1 and USF2, but not the truncated NRSF isoform sNRSF.

4.4.2 The TAC1 proximal promoter contains a -60 E box motif, which acts as an enhancer in SK-N-AS cells.

The rat TAC1 promoter spanning -865 +92 (-865 TAC1) contains a functional E box motif (5'-CAGCTG-3') at -60 relative to the major TSS, known hereafter as the -60 E box. This -60 E box has been shown be important in supporting TAC1 promoter activity in rat PC12 cells (Paterson *et al.,* 1995) and is conserved in the human TAC1 promoter. I validated that in human SK-N-AS neuroblastoma cells, the rat TAC1 (-865 TAC1) promoter supported high levels of luciferase expression and that disruption of the -60 E box site via a 10bp insertion (as in the -60 TAC1 reporter gene construct) resulted in a significant 80% reduction in reporter gene activity ($P = < 0.01$) (n=18) (Figure 4.3), thus demonstrating that the -60 E box is a major determinant of TAC1 promoter activity in SK-N-AS cells.

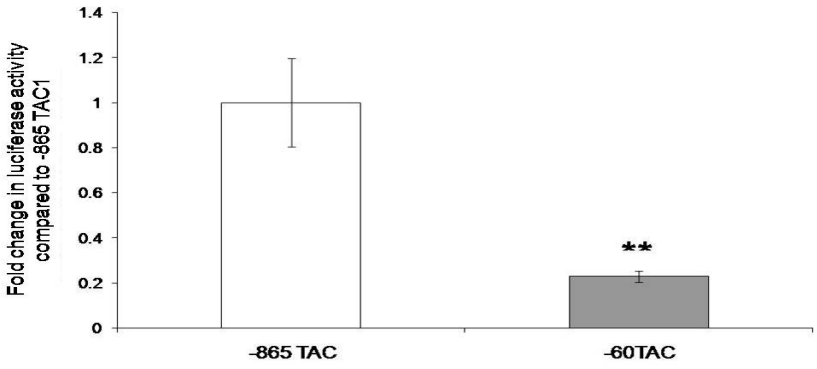

Figure 4.3 -60 E box site acts as an enhancer. Comparison of the -865 TAC1 and -60 TAC1 reporter gene constructs luciferase activity, reveals the -60 TAC mutant construct has significantly lower activity compared to -865 TAC1 construct, indicating that the -60 E box as an enhancer. Human SK-N-AS cells were transfected with either the -865 TAC or the -60 TAC1 reporter gene constructs and luciferase activity was measured, with mean fold changes compared to untreated -865 TAC1 given. Statistics were performed using *T*-test, with ** = $P < 0.01$. S.E. are given as Y-axis error bars (n=6, performed in triplicate).

4.4.3 Modulation of TAC1 promoter by Upstream Stimulatory Factors (USF) 1 & 2

The role of USF1 and USF2 in the regulation of the TAC1 promoter was investigated by analysing the response of both the -865 TAC1 and -60 TAC1 mutant reporter gene constructs, to the over-expression of USF1 and USF2 in human SK-N-AS cells. Over-expression of USF1 or USF2, had no significant impact on -865 TAC1 reporter gene activity, compared to untreated basal reporter gene levels ($P > 0.05$) (n=12) (Figure 4.4a). In contrast, the activity of the -60 TAC1 mutant, was significantly elevated 3-fold by both USF1 and USF2 ($P = < 0.01$) (n=12) over-expression (Figure 4.4b). One is therefore left to assume that this increase in reporter gene expression is via one or more of the other E boxes in this promoter construct. However it should be noted that the increase in -60 TAC1 mutant reporter gene activity with USF over-expression, reaches a similar level to the wild type (-865 TAC1) promoter alone (Figure 4.4b) ($P > 0.05$) (n=12). This may indicate that USF1 and USF2 may stimulate activity of the TAC1 promoter via different E box sites, negating the loss of the -60 E box site, and returning the activity of the TAC1 promoter back to the wild type.

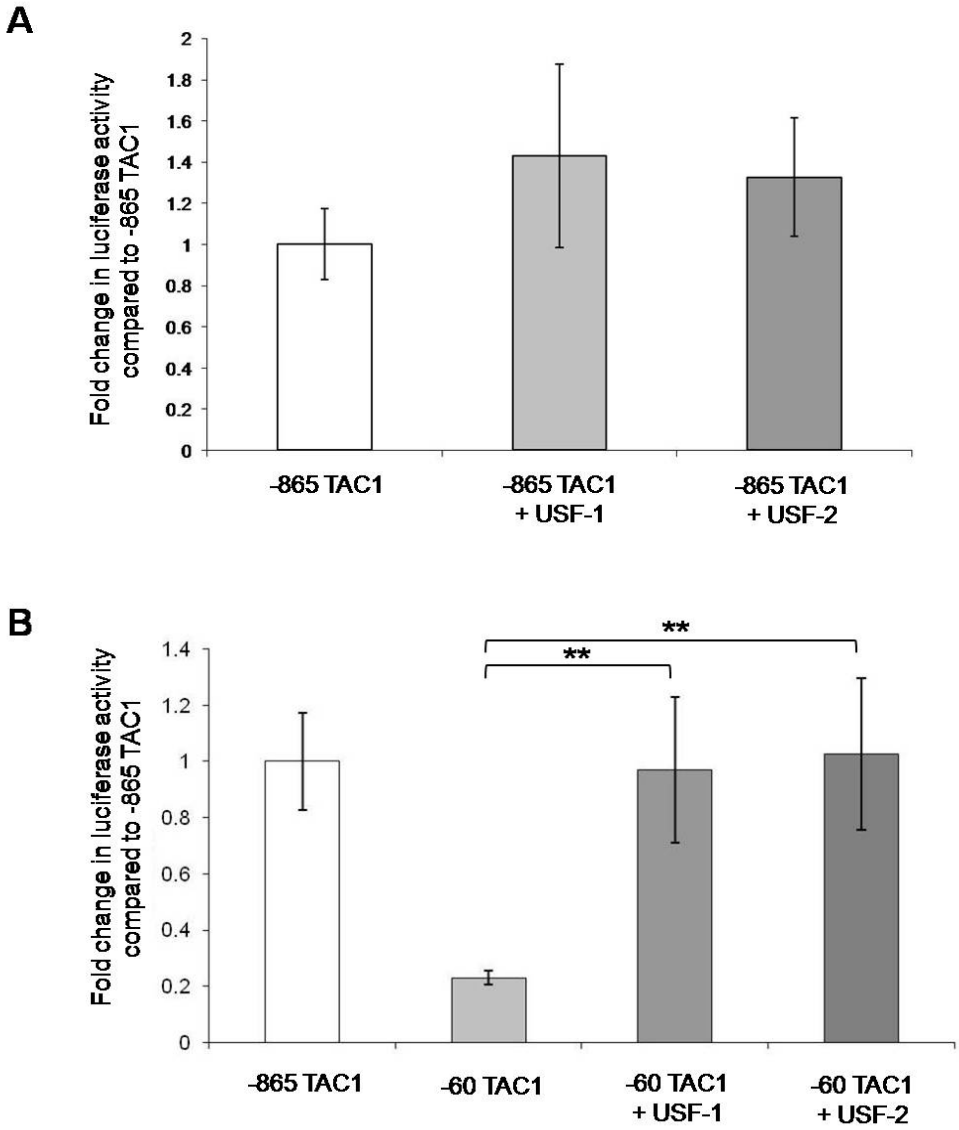

Figure 4.4. Regulation of rat TAC1 reporter gene activity by USF1 & USF2 in SK-N-AS cells. (A) Over-expression of USF1 and USF2 expression constructs had no significant impact on the -865 TAC reporter gene construct activity. **(B)** In contrast both USF1 and USF2 led to significant increase in -60 TAC reporter gene activity, elevating activity to the same level as the -865 TAC construct alone. Human SK-N-AS cells were transfected with -865 TAC reporter gene construct and the USF1 or truncated USF2 expression constructs. Luciferase activity was measured and mean fold changes compared to basal are given. Statistics were performed using T-test, with ** = $P < 0.01$. S.E. are given as Y-axis error bars (n=4, performed in triplicate).

4.4.4 Regulation of TAC1 promoter by the NRSF isoforms is independent of the -60 E box.

The over-expression of both the full length NRSF (REEX1), or a truncated variant (HZ4), resulted in increased reporter gene expression supported by the TAC1 promoter in human SK-N-AS cells, similar to previous findings in rat hippocampal cultures (Spencer *et al*., 2006). NRSF was found to act as an activator of the -865 TAC promoter activity, with REEX1 over-expression resulting in a 2.5x-fold increase in promoter activity (Figure 4.5a) ($P = < 0.01$) (n=12). This increase was found to be independent of the -60 E box site, as disruption of this site (-60 TAC1 mutant) gave the same 2.5-fold increase in reporter gene construct activity compared to basal (Figure 4.5b) ($P = < 0.01$) (n=12). Similarly over-expression of the HZ4 construct resulted in a significant 2x fold increase in -865 TAC1 reporter gene activity (Figure 4.5a) ($P = < 0.01$) (n=12). As with NRSF, this increase in reporter gene expression following HZ4 over-expression was found to be independent of the -60 E box site (Figure 4.5b) ($P = < 0.01$) (n=12). Furthermore, in our cell line model, although not conclusive, both NRSF variants, generate the same 2-2.5x fold increase in TAC1 activity, suggesting that potentially the shared N-terminal region of NRSF, common to both REEX1 and HZ4 is responsible for the regulation of TAC1.

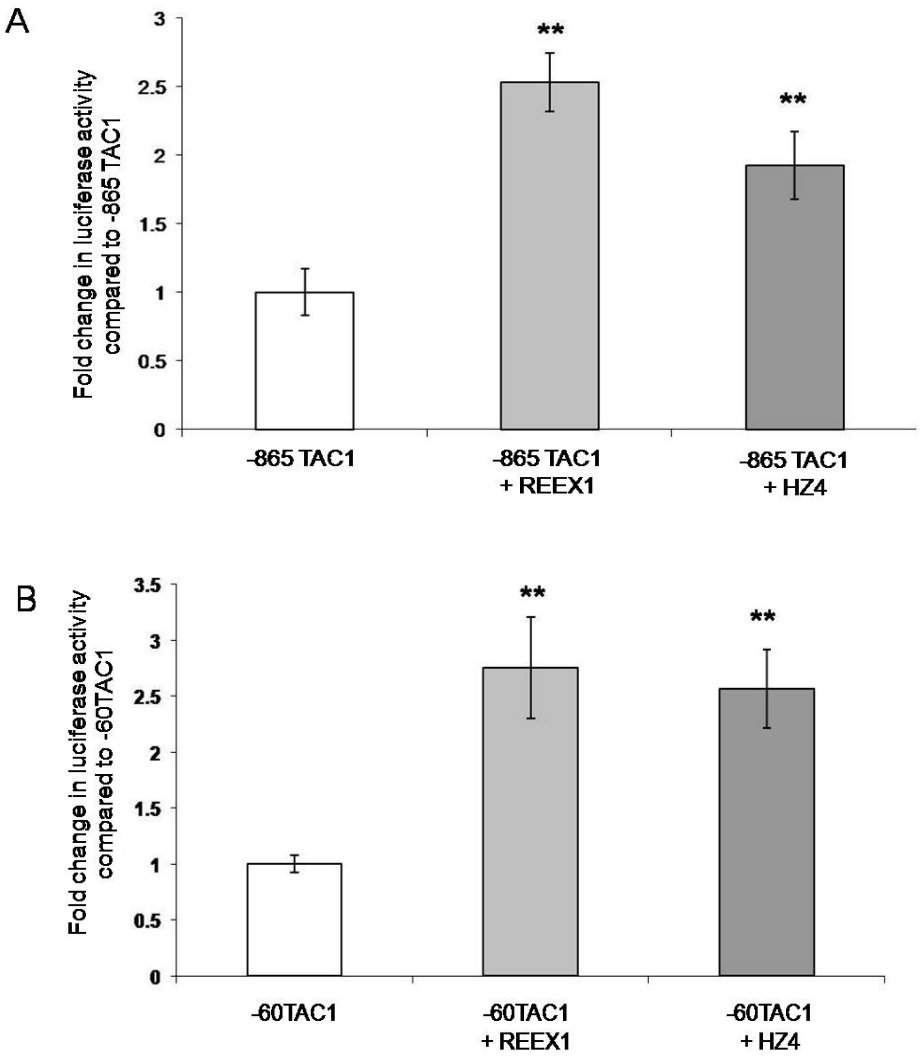

Figure 4.5. Regulation of TAC1 reporter gene activity by NRSF variants in SK-N-AS cells. Over-expression of both full-length NRSF (REEX1) and a truncated construct analogous to sNRSF (HZ4) caused a significant increase in both **(A)** -865 TAC and **(B)** -60 TAC reporter gene construct activities. Human SK-N-AS cells were co-transfected with either the -865 TAC or -60 TAC reporter gene constructs and the full length NRSF (REEX1) or truncated sNRSF (HZ4) expression constructs. Luciferase activity was measured and mean fold changes compared to reporter gene construct alone are given. Statistics were performed using Students *T*-test, with ** = $P < 0.01$. S.E. are given as Y-axis error bars (n=4, performed in triplicate).

4.4.5 NRSF variants are capable of enhanced activation of the TAC1 promoter in conjunction with USF, but via different mechanisms.

The data presented in this chapter demonstrates that both USF and NRSF proteins are important in the regulation of the rat TAC1 promoter. Similarly, in chapter 3, I demonstrate important regulatory roles for both USF and NRSF proteins in the regulation of the related tachykinin gene TAC3, and our group has also indicated a role for both of these TFs in the regulation of AVP (Coulson *et al*., 1999; Quinn *et al*., 2002; Coulson *et al*., 2003). As previously indicated in chapter 3, the position of the NRSE in all three neuropeptide genes is close to the TSS and that all three gene promoters contain E box motifs in close proximity to their NRSEs. Thus, I was interested in exploring the possibility of an inherent mechanistic relationship between NRSF and USF.

To investigate this, SK-N-AS cells were transfected with either REEX1 or HZ4 plus either USF1 or USF2 expression constructs, together with the TAC1 reporter gene constructs. Co-expression of NRSF (REEX1) plus either USF1 or USF2, failed to have an impact on TAC1 regulation, when compared to REEX1 alone (Figure 4.6a) ($P = >0.05$) (n=12). In contrast co-expression of the truncated variant, (HZ4) plus USF1 (3.6x fold compared to -865 TAC1 alone) and USF2 (3.3x fold compared to -865 TAC1 alone) led to a significant additive increase in -865 TAC1 promoter activity compared to single treatment with HZ4, USF1 or USF2, alone (1.9x fold, 1.4x fold and 1.3x fold compared to -865 TAC1 alone, respectively) (Figure 4.7a) ($P = < 0.01$) (n=12).

To determine the potential mechanisms underlying the observed differences between the NRSF variants, I performed the same co-transfections with the (disrupted) -

60 E box construct. In direct contrast to the impact upon the wild type -865 TAC1 promoter, co-expression of NRSF (REEX1) plus USF1 or USF2, led to a significant increase in the reporter construct activity of the -60 TAC1 mutant (6.3x fold and 8.9x fold, compared to -60 TAC1 alone, respectively), when compared to treatment with either NRSF, USF1 or USF2 alone (2.7x fold, 3.1x fold and 3.2x fold, compared to -60 TAC1 alone, respectively) (Figure 4.6b) ($P = < 0.01$) (n=12). Interestingly, the co-expression of HZ4 plus USF1 or USF2 had no impact on the -60 TAC1 mutant promoter activity (Figure 4.7b) ($P > 0.05$) (n=12), indicating that the additive affect of HZ4 plus USF is dependent on the -60 E box site.

4.4.6 USF binding to the human TAC1 promoter is cell specific.

In chapter 3, USF binding to the human NKB promoter was found to differ between sNRSF expressing (SH-SY5Y) and non-expressing (SK-N-AS) cells. Based upon findings in this chapter indicating a cooperative regulatory role between NRSF variants and USF proteins, I was interested in characterising NRSF and USF binding to the endogenous TAC1 promoter in the human cell lines employed. In the sNRSF non-expressing SK-N-AS cell line, ChIP assays revealed that both USF1 and USF2 were bound to the human TAC1 promoter locus, when compared against the non-specific background (negative) control; IgG (Figure 4.8a). In addition, NRSF was found to be bound to human TAC1 promoter locus, but as expected, sNRSF was not bound (Figure 4.8a). Interestingly, in the sNRSF expressing cell line, SH-SY5Y, USF1 binding is lost, with only NRSF, sNRSF and USF2 found bound to the human TAC1 promoter locus (Figure 4.8b). This is consistent with the lack of USF1 binding observed at the human NKB promoter locus in SH-SY5Y cells, as described in Chapter 3.

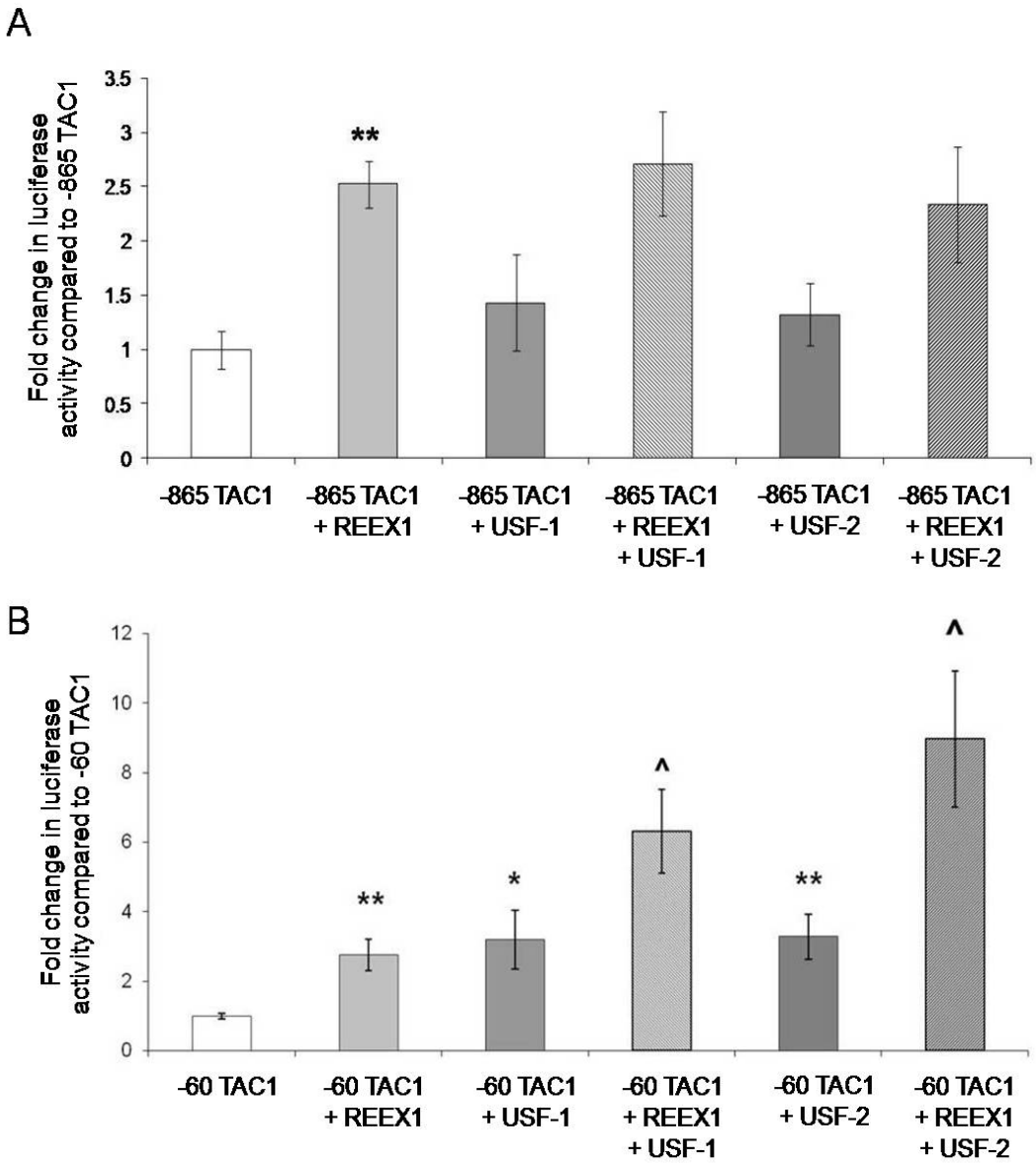

Figure 4.6. Additive regulation by NRSF & USF requires the disruption of the -60 E box. (A) Combined over-expression of NRSF (REEX1) together with either USF1 or USF2 had no impact on -865 TAC reporter gene activity when compared to the affect of REEX1 alone. **(B)** In contrast, enhanced -60 TAC reporter gene activity was observed following combined over-expression of REEX1 with USF1 or USF2. Human SK-N-AS cells were co-transfected with **(A)** -865 TAC or **(B)** -60 TAC reporter gene constructs, together with the NRSF (REEX1), USF1 or USF2 expression constructs, singularly and in cotransfection combination. Luciferase activity was measured and mean fold changes compared to basal are given. Statistics were performed using student's *T*-test, with * = $P < 0.05$, ** = $P < 0.01$ (basal vs. single transfection) and ^^ = $P < 0.01$ (co-transfection vs. single transfection). S.E. are given as Y-axis error bars (n=4, performed in triplicate).

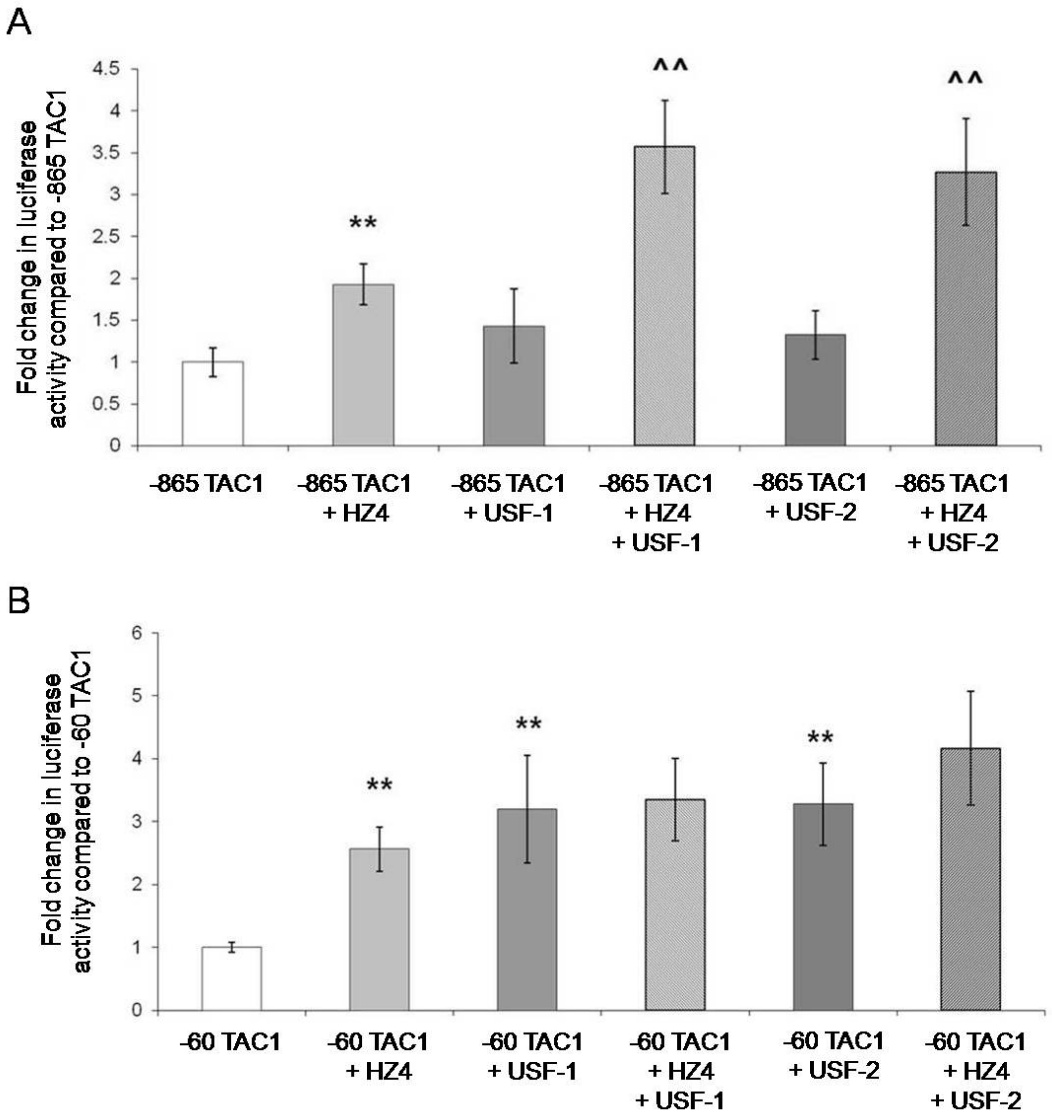

Figure 4.7. Additive regulation by HZ4 & USF requires the -60 E box. (A) Combined over-expression of both the truncated construct HZ4 together with either USF1 or USF2 expression constructs resulted in a significant increase in -865 TAC reporter gene construct activity when compared to single transfection of HZ4 alone. **(B)** In contrast, combined over-expression of both HZ4 together with either USF1 or USF2 expression constructs had no significant impact on the -60 TAC reporter, compared to the single transfection of HZ4 alone. Human SK-N-AS cells were co-transfected with **(A)** -865 TAC or **(B)** -60 TAC reporter gene construct, together with the truncated NRSF construct (HZ4), USF1 or USF2 expression constructs, singularly and in co-transfection combination. Luciferase activity was measured and mean fold changes compared to basal are given. Statistics were performed using student's *T*-test, with * = $P < 0.05$, ** = $P < 0.01$ (basal vs. single transfection) and ^^ = $P < 0.01$ (co-transfection vs. single transfection). S.E. are given as Y-axis error bars (n=4, performed in triplicate).

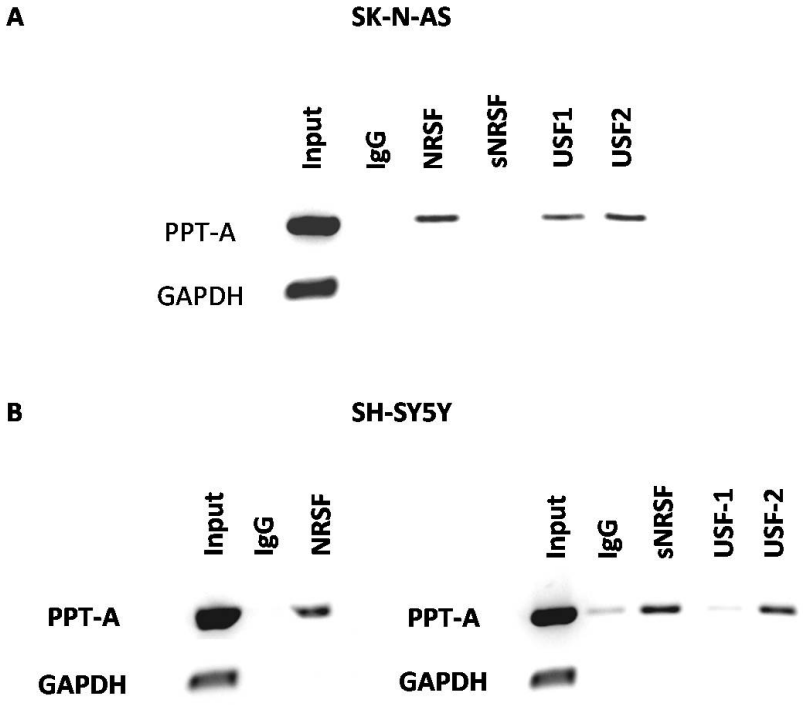

Figure 4.8. NRSF and USF binding to the human TAC1 proximal promoter. (A) ChIP analysis revealed that NRSF, USF1 and USF2 show binding to the human TAC1 promoter in untreated human SK-N-AS cells, compared to IgG negative control. The truncated isoform sNRSF was as expected, not found to bind.**(B)** In sNRSF expressing SH-SY5Y cells, NRSF, sNRSF and USF2 were found to bind to the human TAC1 promoter locus. In all cases, binding is shown to be specific to the human TAC1 proximal promoter, as no binding is observed in the GAPDH PCR negative controls (n=1).

4.5 Discussion

The TFs NRSF and USF have been shown by our lab and others to regulate a small set of common target genes including AVP (Coulson *et al.,* 1999), BDNF (Tabuchi *et al*., 2002a) and NKB (chapter 3). In this chapter I have confirmed the role of both NRSF variants and USF proteins in the regulation of the TAC1 promoter, as shown by our group and others (Quinn *et al.,*2002;Spencer *et al.,* 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009; Paterson *et al*., 1995; Gerrard *et al*., 2005). Due to the close proximity of the NRSE to the TSS and to characterised or putative E box motifs, within the neuropeptides AVP, TAC3 and TAC1 promoters, I was interested in exploring a potential inherent mechanism existing between NRSF and USF in the regulation of such neuropeptide genes. In this chapter I show that the NRSF variants can differentially enhance TAC1 promoter activity in conjunction with USF proteins, dependent, in part, on the integrity of an E box motif at -60 relative to the major TSS.

The E box motif at -60 was found to act as an enhancer in human SK-N-AS cells. The rat promoter spanning -865 to +92 (-865 TAC1) supported high levels of luciferase activity, which was significantly reduced 5x fold, following the abolishment of the -60 E box (-60 TAC1). This is consistent with previous findings from our group in rat PC12 cells (Paterson *et al*., 1995). Interestingly, the over-expression of USF proteins failed to have an impact on the wild type rat TAC1 promoter (-865 TAC1), but did induce a 3x fold increase in luciferase supported by the mutant -60 TAC1 construct. Simplistically this indicates that USF1 and USF2 can modulate the activity of the TAC1 promoter through other E box motifs within the proximal promoter, as the abolishment of the -60 E box could inhibit USF recognition and binding to this site. Interestingly, the

elevation in activity of the -60 construct failed to surpass the basal level of the wild type -865 TAC1 reporter gene construct. This suggests that whilst USF1 and USF2 are capable of modulating TAC1 promoter activity through different E box motifs, the -60 E box may still remain the most important element, as other E box motifs cannot drive expression higher than the basal wild type -865 TAC1 construct in response to USF. These findings indicate that the -60 E box cannot wholly account for any USF-mediated modulation of TAC1 promoter, and implies that other E boxes are also important in regulating the TAC1 promoter.

Regulation of the rat TAC1 promoter by NRSF variants (alone) was found to be independent to the -60 E box motif, as both the -865 TAC1 and -60 TAC1 responded in the same way to full length (REEX1) over-expression, and to that of a truncated construct analogous to the truncated NRSF variant, sNRSF (HZ4). This is, in part, consistent with previous finding from our group, in that REEX1 over-expression drives elevated luciferase activity supported by the rat TAC1 promoter in cultured hippocampi (Spencer *et al*., 2006). However, in these hippocampal cultures, the HZ4 construct was found to drive x3 fold higher luciferase activity compared to REEX1 over-expression (Spencer *et al*., 2006), which is not seen here.

Over-expression of HZ4 together with either USF1 or USF2 was shown to drive luciferase activity supported by the wild type -865 TAC1 promoter, approximately 2x fold higher than compared to HZ4, USF1 or USF2 alone. This additive activity was found to be dependent on the -60 E box, as abolishment of this motif (in the -60 TAC1 construct), led to no significant impact on luciferase activity when over-expressing HZ4 with either USF constructs, compared to HZ4, USF1 or USF2 alone. In contrast, over-

expression of full-length NRSF via the REEX1 construct, failed to drive enhanced luciferase activity of the wild type -865 TAC1 promoter, when in conjunction with USF1 or USF2, compared to REEX1, USF1 or USF2 alone.

This is interesting as, in this chapter, I show that over-expression of HZ4, and not REEX1, has an additive impact on luciferase activity of the wild type -865 TAC1 construct, when over-expressed in conjunction with USF1 or USF2. This could be of particular relevance to TAC1 regulation during seizure. NRSF isoforms are known to be up-regulated during seizure (Palm *et al*., 1998, Spencer *et al*., 2006), and whilst there are no publications regarding USF modulation during seizure, USF proteins are known to be expressed in the CNS (Corre & Galibert., 2005). Therefore it is plausible that seizure-induced up-regulation of NRSF isoforms, induces enhanced TAC1 regulation, and when USF proteins are also present, TAC1 regulation is further enhanced by coordinated regulation between USF and truncated NRSF isoform (summarised in Figure 4.9). This is supportive of a hypothesis that it is the truncated NRSF isoform, which is of greatest importance in the regulation of TAC1 in KA seizure models (Spencer *et al*., 2006), as the full-length NRSF fails to regulate the wild-type TAC1 promoter in cooperation with the USF proteins, and so does not have the same degree of regulatory control over the TAC1 promoter, as the truncated isoform.

Interestingly, in Chapter 6, I show that KA does induce elevated USF mRNA expression in human neuroblastoma cells, thus it would be of great interest to monitor USF expression during an *in vivo* rodent seizure model, to establish the affects of seizure on USF expression. Furthermore, it would be of interest to monitor endogenous TAC1 mRNA expression in response to both HZ4 and USF over-expression, singularly

and in combination, in cell lines and *in vivo*, to explore whether or not this cooperative regulation of the TAC1 promoter, has an effect on endogenous expression.

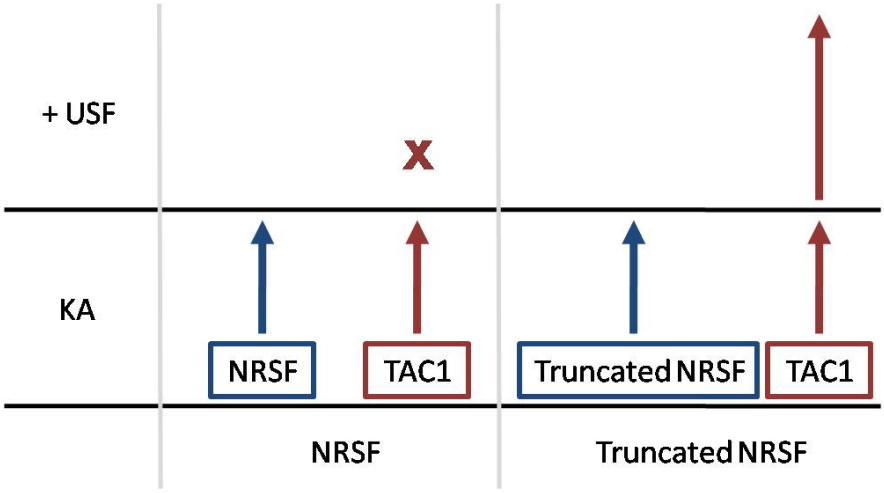

Figure 4.9 Potential significance of USF and truncated NRSF cooperative regulation of TAC1. During KA-induced seizure, both full-length and truncated NRSF isoforms are up-regulated, which drives an increase in TAC1 expression (Spencer *et al*., 2006). USF proteins can additively regulate the wild-type rTAC1 promoter in cooperation with the truncated variant only, which is supportive of the theory that the truncated NRSF variant is of greater importance in seizure-induced TAC1 regulation.

Clues to how the truncated isoform and USF proteins could facilitate and enhance TAC1 promoter activity may come from studying the TF binding profile to the endogenous TAC1 promoter in humans. The human TAC1 promoter shares a degree of homology with the rat promoter, with the three E box motifs located at -60, -170 and -308 in the rat, perfectly conserved in the human, with the closest E box within 34bp of a putative NRSE (based upon sequence alignment against the rat TAC1 NRSE) (See Appendix 2, 3 & 4), similar to the rat, whereby the rat -60 E box is within 33bp of the characterised NRSE (See Figure 4.1 & Appendix 4). In the human SK-N-AS cell line, the truncated isoform sNRSF is absent. In these (untreated) cells, the preliminary ChIP experiment, suggested that both USF1 and USF2 could bind to the human TAC1

promoter locus, as could full-length NRSF, whereas, as one might expect, sNRSF was not found to be bound to that region. Interestingly, in untreated sNRSF expressing cells (SH-SY5Y), sNRSF was found to bind to the human TAC1 promoter locus, at the expense of USF1, with USF1 no longer bound (but USF2 and NRSF remained bound).

USF1 and USF2 predominantly form heterodimers (Corre & Galibert., 2005), and so one may expect to find both bound to the same DNA locus. In the SK-N-AS cell line, both USF1 and USF2 were found bound to the TAC1 promoter region. The presence of sNRSF, may in some way abolish USF1 binding to the human TAC1 promoter locus, and this pattern in consistent with that observed in chapter 3 at the human NKB locus. This may suggest that sNRSF competes with USF1 for either binding privileges at the TAC1 and NKB promoter. The fact that abolishment of the -60 E box in the rat TAC1 promoter, which is the closest E box to the rat NRSE, inhibits sNRSF and USF cooperative regulation, may be supportive of a model in which sNRSF and USF interact. The rat NRSE is within 50bp of the -60 E box, and this close proximity may lead to some interaction between sNRSF and USF. Disrupting this E box would prevent USF binding, preventing any possible interactions, and thus abolishing the cooperative regulation. Additional support for NRSF and USF interactions have come from unpublished findings from our group in which complexes bond to the rat TAC1 NRSE (NRSF/sNRSF) were found to be removed by homologous competitors in electophoretic mobility shift assays (EMSAs), as expected, but also by a -60 E box specific competitors (USFs) (Spencer *et al*., thesis). This suggested that proteins bound to the rat TAC1 NRSE could bind to, or interact with those which bind to the -60 E box (USF) (Spencer *et al*, thesis 2006). Subsequently, efforts were made to explore potential

interactions between the NRSF isoforms and USF1 and USF2, through Co-IP, but unfortunately the technique was not optimised in time for this thesis. This however remains an important experiment to perform, to fully support this interaction theory.

NRSF was also found to work in cooperation with the USF TFs, but only when the -60 E box was disrupted. Over-expression of full-length NRSF (REEX1) in combination with USF had no impact on the wild type -865 TAC1 promoter, but did lead to an additive increase in luciferase activity supported by the disrupted -60 E box mutant -60 TAC1. This suggests that NRSF and USF have the capacity to cooperate to enhance TAC1 regulation, but requires USF to bind to an E box other than the -60 E box. USF over-expression failed to modulate the wild type -865 TAC1 construct, but did activate the -60 TAC1 construct, indicating that USF could function via different E box sites. Therefore, potential cooperation between NRSF and USF may exist. It would be thus of interest to address NRSF and USF interactions (via Co-IP), and characterise USF binding to other E box motifs, perhaps via the generation of multiple mutant constructs, one per E box, to elucidate the importance of each on TAC1 regulation.

The differences observed in this chapter, are often difficult to explain, but what is clear is that NRSF isoforms are capable of regulating the TAC1 promoter in conjunction with USF proteins, in a differential manner, and that this is, in part, dependent on the key -60 E box motif.

CHAPTER 5: NRSF isoforms are modulated by ACD treatment

5.1 Introduction

The importance of the NRSF TF family in regulating the pro-convulsant tachykinin genes TAC3 and TAC1 has been discussed in Chapters 3 and 4, respectively. NRSF has also been shown to regulate a host of neuronal genes with known or suggested roles in epilepsy (as further explored and discussed in Chapter 7), such as BDNF (Timmusk *et al*., 1999; Koyama & Ikegaya., 2005; Hara *et al*., 2009), and has also been implicated in the molecular mechanisms governing the anti-epileptic ketogenic diet (Garriga-Canut *et al*., 2006), in which NRSF mediated repression of BDNF, and its receptor TrkB, is enhanced following glycolytic inhibition. Due to these findings and the fact that NRSF isoforms are modulated in KA epilepsy models (Palm *et al.,* 1999; Spencer *et al.,* 2006), I sought to investigate whether or not NRSF isoforms were targets for ACD modulation.

A recent study found that the ACD Valproic acid (VPA), a known HDAC inhibitor, can reduce the expression of full-length NRSF, whilst up-regulating both BDNF and TrkB (Kim *et al.,* 2007), thus indicating a possible role of ACDs in modulating NRSF expression. Furthermore, both CBZ and LMT have been shown to up-regulate BDNF expression in the rat cortex (Chang *et al,*2009), suggesting these ACDs can also target the NRSF regulatory system in a similar manner. In this study, I have opted to focus on CBZ, LMT and a third ACD, PHY. These three drugs are widely prescribed in the UK for the treatment of epilepsy, and share similar mechanisms of action as they are known to function via sodium channel blockade (Reviewed by

Rogawski & Loscher., 2004a), by binding to a common recognition site on the sodium channel (Kuo., 1998). Perhaps due to this common trait, all three drugs are found to be beneficial for the same type of epilepsies (GTC and Partial seizures) (Reviewed by Rogawski & Loscher., 2004a).

However, despite being used in the treatment of both GTC and partial seizures and sharing the same sodium channel target, these three ACDs each have different properties and can elicit different cellular responses. In the first instance, the three drugs have different structures, with PHY being a hydantoin molecule containing the ureide structure, which is traditionally viewed as a key structure for anti-convulsant activities. CBZ in contrast, is a tricyclic molecule, with a short amide side chain, missing the ureide structure, whilst LMT is the smallest compound containing just two aromatic rings (Kuo., 1998). Secondly, with regards to differential clinical uses, it is known that all three drugs have reported mood stabilising properties, but are used differently in the clinic, with CBZ and PHY routinely used in the treatment of the manic phase of BD, whereas, LMT is used to treat the depressed phase of BD (Reviewed by Rogawski & Loscher., 2004b). LMT has also been shown to be useful in the treatment of Absence seizures and Myoclonic seizures (Posner *et al.,* 2005; Wheless *et al.,* 2007; Auvin., 2008), and this is perhaps due to LMT targeting high voltage calcium channels (Stefani *et al.,* 1996; Wang *et al*., 1996), which are known to be crucial for abnormal oscillatory behaviour underlying generalized absence seizures (Huguenard *et al*., 1996).

It is therefore anticipated that by exploring these three different ACDs, and comparing the outcome of each drug treatment individually on the NRSF regulatory system, one may reveal the underlying molecular mechanisms of seizure generation and

progression and perhaps gain a greater understanding of how else these drugs elicit their anti-convulsant properties. Our lab has previously revealed that pro-convulsive (KA) treatment has a marked impact upon the expression and localisation of the NRSF isoforms in rat hippocampi, with elevated expression of both full-length and truncated NRSF observed following KA. Furthermore, KA also induced a switch in the cellular localisation of both isoforms, with NRSF translocating to the nucleus whilst the truncated isoform moved into the cytoplasm (Spencer *et al*., 2006). Here I aim to explore the impact of ACD treatment on both the expression levels and localisation of the NRSF isoforms, in human neuroblastoma cells. In addition, the impact of ACD treatment on NRSF binding to its 21bp recognition sequence, the NRSE, in a number of relevant NRSF regulated genes, will be explored to determine whether or not ACDs can impact upon NRSF function, in addition to modulating expression or localisation.

5.2 Aims

- To test if pro-convulsant treatment (KA) modulates the mRNA expression of the NRSF isoforms in human neuroblastoma cells (SH-SY5Y).
- To explore the impact of ACD treatment on NRSF isoform mRNA expression in SH-SY5Y cells, using the clinically prescribed ACDs CBZ, PHY and LMT.
- To explore NRSF isoform localisation in human neuroblastoma cells.
- To determine if the ACDs affect NRSF isoform localisation.
- To determine NRSF binding to target NRSE-containing regions in SH-SY5Y cells via ChIP.
- To explore the affects of ACD treatment on NRSF binding to regions containing target NRSEs.

5.3 Methods

5.3.1 Cell culture and treatment

Human SH-SY5Y cells were used throughout as RT-PCR revealed they express both full-length NRSF and the truncated isoform sNRSF (see chapter 3, Figure 3.1). In addition, the TAC1 expressing SK-N-AS cell line was used in a single experiment to monitor ACD modulation of TAC1 expression. SH-SY5Y and SK-N-AS cells were cultured as outlined in methods section 2.2.2.1.1 and 2.2.2.1.2, respectively, and treated with KA, ACDs or vehicle controls as described in section 2.2.2.2. KA, ACDs and the corresponding vehicle controls were prepared as described in section 2.1.3. For mRNA analysis, cells were then harvested as detailed in methods section 2.2.3.1, 2.2.3.3 and measured as described in section 2.2.3.5. For immunofluorescence, cells were fixed and processed as described in methods section 2.2.4.5 and for ChIP assays, cells were fixed and protein/DNA interactions cross-linked as described in section 2.5.1.

5.3.2 mRNA expression analysis

The expression of the TFs NRSF and sNRSF, as well as the house keeping gene Pol II, was investigated using qPCR as described in methods section 2.2.3.5, using the PCR primers set out in Table 2.2.1. Thermal cycle conditions for NRSF and Pol II, were as follows: initial denaturation: 95^{o}C for 3 minutes for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds and annealing 62^{o}C for 30 seconds; and a final elongation step of 72^{O}C for 2 minutes. For sNRSF the annealing temperature was reduced to 58.5^{o}C, with all other conditions remaining the same. Specificity of products

was determined by subsequent melt curve analysis from 55°C to 95°C increasing in 0.5°C increments

5.3.3 Protein analysis

5.3.3.1 Western Blotting

The anti-NRSF (R2174) and anti-rREST4 (R3122) antibodies were used on protein extracted from untreated SH-SY5Y cells as described in section 2.2.4.1. Western blotting was performed as outlined in methods section 2.2.4.3.

5.3.3.2 Immunofluorescence

SH-SY5Y cells cultured on cover-slips as described in section 2.2.2.1.2, treated as in section 2.2.2.2, and harvested as described in section 2.2.4.4. Cover-slip mounted cells were probed with antibodies described in Table 2.3, and mounted and visualised as outlined in section 2.2.4.4.

5.3.4 TF binding study - ChIP

To investigate NRSF TF binding to regions encompassing characterised and putative NRSEs, ChIP was employed as outlined in method section 2.2.5, using the antibodies given in Table 2.3 and the PCR primers given in Table 2.4.

5.4 Results

5.4.1 Modulation of NRSF isoform mRNA following KA treatment.

In order to test the impact on NRSF isoforms of ACD treatment, a cell line responsive to pro-convulsive stresses was required. In chapter 3, the human neuroblastoma cell line SH-SY5Y was shown to express both NRSF isoforms and so I sought to test if NRSF isoforms were modulated following KA treatment in these cells. SH-SY5Y cells were exposed to either 1µm or 5µM KA, or vehicle control, for either 4hrs or 24hrs, before being processed for RNA extraction. NRSF and sNRSF mRNA levels were quantified via qPCR and standardised relative to Pol II.

Modulation of both NRSF isoforms' mRNA expression levels were observed following KA treatment, with 24hrs 1µM KA and 5µM KA inducing a 2x-fold ($P = < 0.05$) and 5x-fold increase ($P = < 0.001$) respectively, in full-length NRSF mRNA (Figure 5.1a) (n=3). qPCR also revealed that 24hrs 5µM KA induced a marked increase in the expression of the truncated isoform sNRSF, with a 3x-fold increase observed (Figure 5.1b) ($P = < 0.001$) (n=3). These observed elevations in NRSF isoform expression levels are, in part, consistent with those observed in the rat *in vivo* models (Spencer *et al*., 2006).

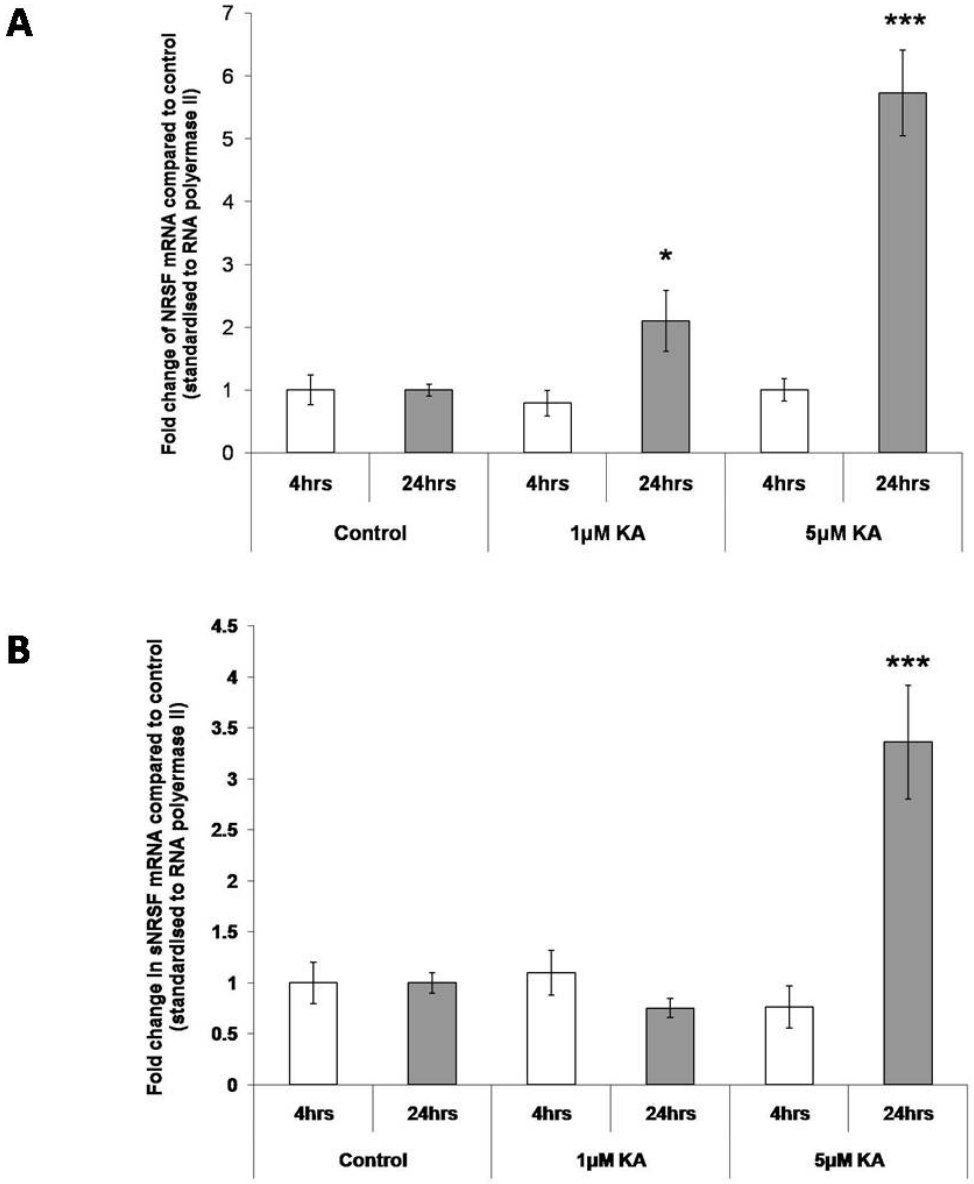

Figure 5.1 Modulation of NRSF isoforms by KA. qPCR analysis of **(A)** NRSF and **(B)** sNRSF mRNA expression in response to KA treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 1µM or 5µM KA, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of NRSF and the truncated isoform sNRSF, were analysed using qPCR, standardized against the house keeping gene Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$; ** = $P = < 0.01$ & *** = $P = < 0.001$. (n=3).

5.4.2 Modulation of NRSF & sNRSF mRNA following ACD treatment.

NRSF isoforms have previously been shown to be modulated during seizure conditions (Palm *et al*., 1998; Spencer *et al*., 2006), and both isoforms are found to be elevated here following KA treatment in SH-SY5Y cells. Due to the importance of NRSF variants in the regulation of the pro-convulsant tachykinins NKB and TAC1 (as discussed in Chapters 3 and 4 respectively), I was interested in exploring the effect of ACD treatment on NRSF and sNRSF mRNA expression. I opted to treat the SH-SY5Y cells with two 1st generation drugs, CBZ and PHY, and a new generation alternative LMT. CBZ, PHY and LMT, are all proposed to function via a similar mechanism of inhibiting voltage-gated sodium channels, but despite this, all three induce distinct patterns of modulation of NRSF isoform (mRNA) expression.

5.4.2.1 CBZ modulation of NRSF & sNRSF mRNA

CBZ treatment was found to induce a time-dependent change in full length NRSF mRNA expression, with 4hrs 10µg/ml CBZ inducing a 2x-fold increase in NRSF mRNA, and 4hrs 50µg/ml CBZ inducing a 4x-fold increase (Figure 5.2a) ($P = < 0.01$) (n=3), when compared to vehicle control (Control C). In contrast, the prolonged exposure led to a significant decrease in NRSF mRNA levels, with both 24hrs 10µg/ml ($P = < 0.001$) and 24hrs 50µg/ml ($P = < 0.01$) inducing approximately a 60% reduction in NRSF mRNA compared to vehicle control (Figure 5.2a) (n=3).

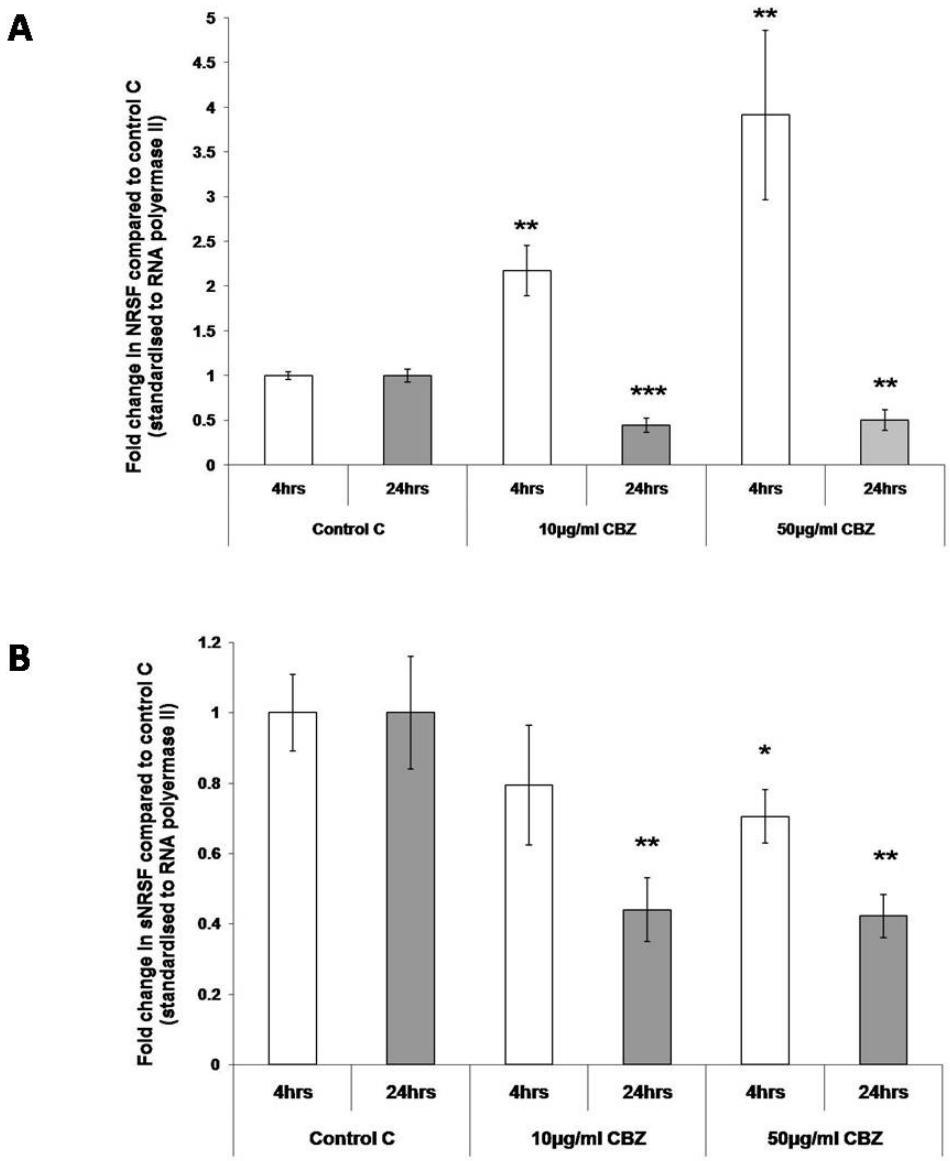

Figure 5.2 Modulation of NRSF isoforms by CBZ. qPCR analysis of **(A)** NRSF and **(B)** sNRSF mRNA expression in response to CBZ treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 10µg/ml or 50µg/ml CBZ, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of NRSF and the truncated isoform sNRSF, were analysed using qPCR, standardized against the house keeping gene Pol II. Mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$; ** = $P = < 0.01$ & *** = $P = < 0.001$.(n=3).

CBZ treatment also led to a significant reduction in the mRNA expression of the NRSF truncated isoform, sNRSF. Following 4hrs CBZ, only the higher 50µg/ml concentration invoked a (30%) reduction in sNRSF mRNA (*P = < 0.05*), compared to vehicle control (Control C). Whereas with 24hrs treatment, both 10µg/ml and 50µg/ml CBZ resulted in a more significant 60% reduction in sNRSF mRNA levels (*P = < 0.01*) (n=3) (Figure 5.2b). It should also be noted that previous studies in our lab have revealed that CBZ and KA treatment (at the concentrations and durations tested here) have no affect on cell viability, with no change in cell death observed (as measured by Propidium Iodide staining) compared to untreated cells (unpublished data). CBZ treatment is therefore of particular interest in modulating the expression of NRSF isoforms, in a time-dependent and concentration-dependent manner.

5.4.2.2 PHY modulation of NRSF & sNRSF mRNA

Despite sharing similar mechanistic actions with CBZ, PHY treatment had no impact on the full-length NRSF mRNA levels, with neither 4hrs nor 24hrs treatment having a significant impact (Figure 5.3a) (*P = > 0.05*) (n=3), when compared to vehicle control (Control P). In contrast, PHY treatment did lead to a significant decrease in the truncated sNRSF isoform mRNA levels, with both 4hrs and 24hrs 50µg/ml PHY (*P = < 0.01 & P = < 0.05,* respectively) inducing approximately a 40-50% reduction in sNRSF mRNA (Figure 5.3b) (n=3).

5.4.2.3 LMT modulation of NRSF & sNRSF mRNA

LMT is one of the newer ACDs, which shares a number of commonalities with CBZ and PHY, in terms of use and mechanism of action, but is believed to have fewer

adverse reactions, based on lower withdrawal from clinical drugs trials of patients taking LMT, compared to those given CBZ (Brodie *et al.,* 1995) or PHY (Steiner *et al.*, 1999). In our SH-SY5Y neuroblastoma cell line, 4hrs LMT induces a significant 60% increase in full-length NRSF mRNA levels, compared to vehicle control (Control L), following both 10µg/ml LMT ($P = < 0.001$) and 50µg/ml LMT treatment ($P = < 0.01$) (Figure 5.4a) (n=3). This increase following 4hrs LMT is similar to that observed following 4hrs CBZ, albeit not as impressive.

As aforementioned, the truncated isoform sNRSF was significantly reduced following both CBZ and PHY treatment. LMT treatment however had no significant impact on sNRSF mRNA levels, with the treatment conditions used here.

5.4.2.4 CBZ modulates TAC1 mRNA

In chapter 4, the importance of NRSF variants on the regulation of the pro-convulsant tachykinin gene TAC1, was discussed. Spencer *et al*, showed that NRSF isoforms are up-regulated following KA stimulation, and this increase coincided with an increase in TAC1 expression at the same time point. Due to the modulation of NRSF isoforms following CBZ treatment, shown in this chapter, I sought to explore the impact of CBZ treatment on endogenous TAC1 expression, in the TAC1 expressing SK-N-AS cell line (See Chapter 4). CBZ treatment was found to induce a significant reduction in TAC1 mRNA levels, following both 10µg/ml and 50µg/ml CBZ, at both 4hrs and 24hrs time points (Figure 5.5) ($P = < 0.01$) (n=3).

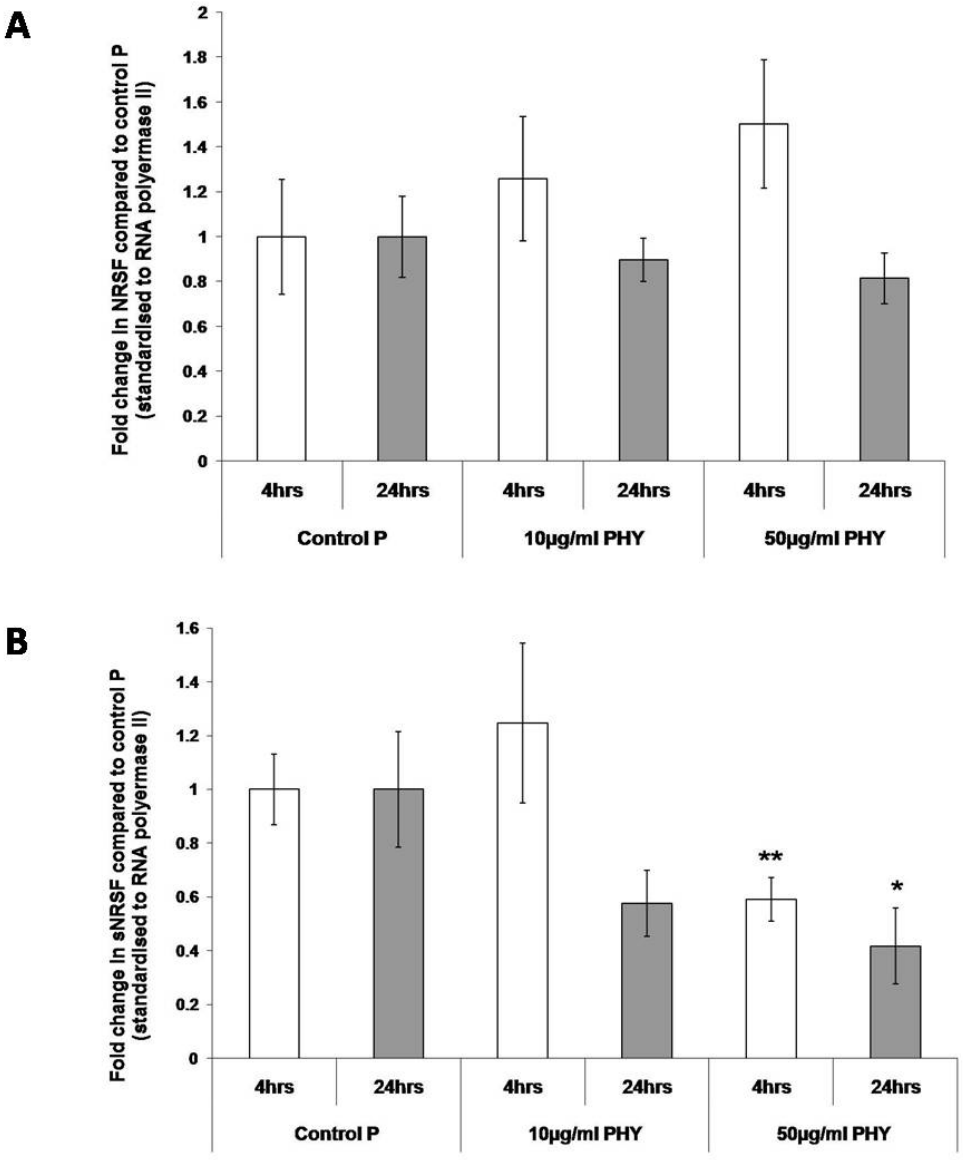

Figure 5.3 Modulation of NRSF isoforms by PHY. qPCR analysis of **(A)** NRSF and **(B)** sNRSF mRNA expression in response to PHY treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 10µg/ml or 50µg/ml PHY, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of NRSF and the truncated isoform sNRSF, were analysed using qPCR, standardized against the house keeping gene Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$; ** = $P = < 0.01$ & *** = $P = < 0.001$. (n=3).

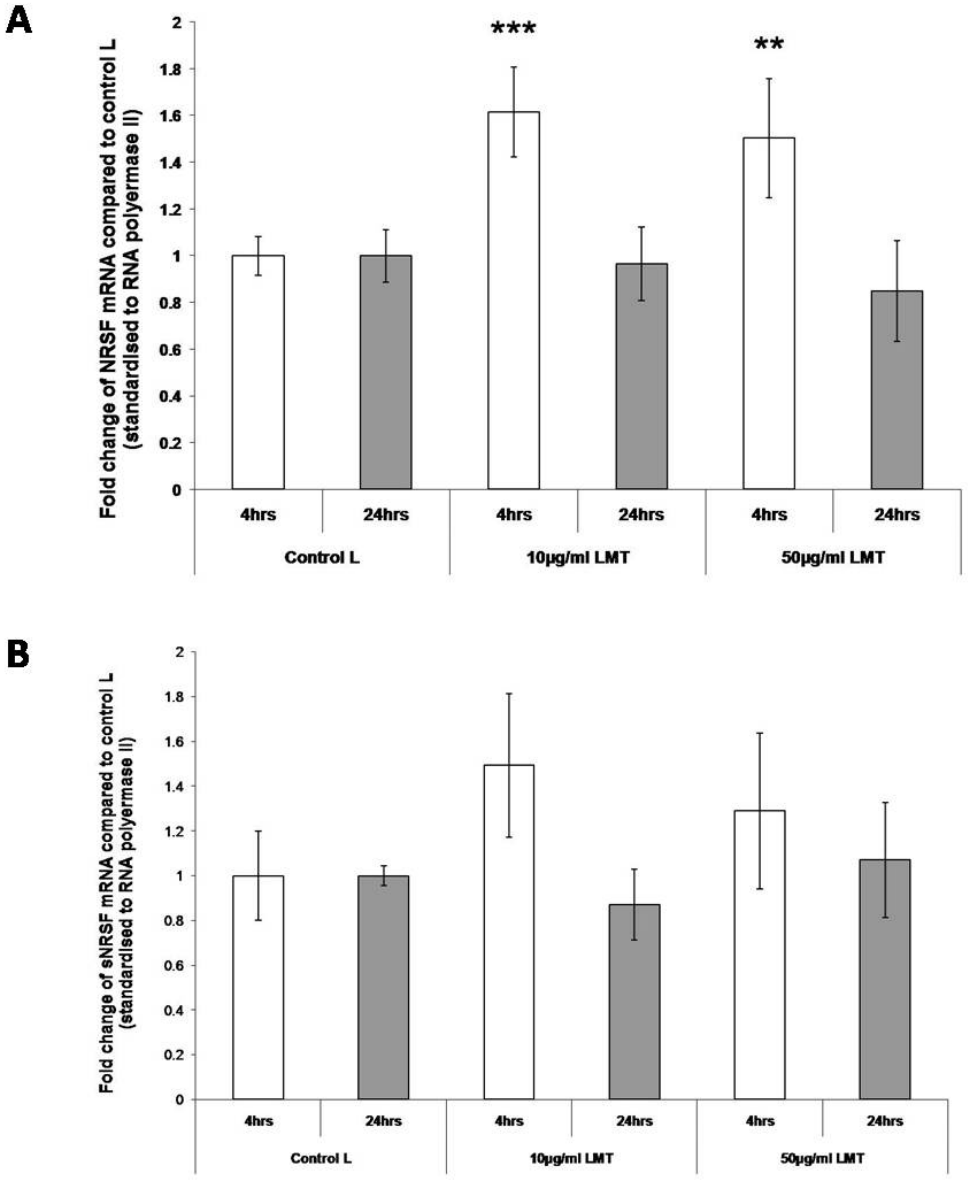

Figure 5.4 Modulation of NRSF isoforms by LMT. qPCR analysis of **(A)** NRSF and **(B)** sNRSF mRNA expression in response to LMT treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 10µg/ml or 50µg/ml LMT, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of NRSF and the truncated isoform sNRSF, were analysed using qPCR, standardized against Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Student's *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$; ** = $P = < 0.01$ & *** = $P = < 0.001$. (n=3).

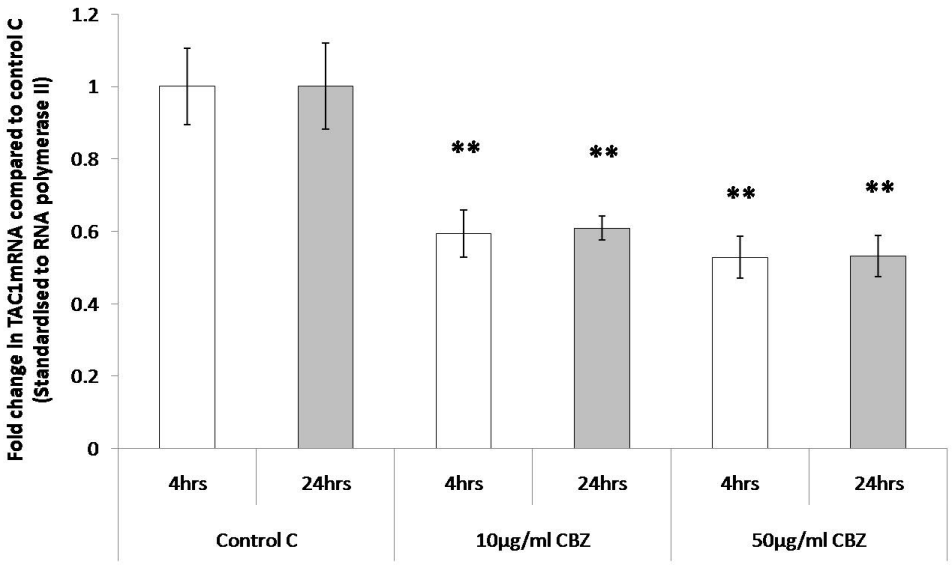

Figure 5.5 Modulation of TAC1 by CBZ. qPCR analysis of TAC1 mRNA expression in response to CBZ treatment in human SK-N-AS neuroblastoma cells. SK-N-AS cells were treated with either 10µg/ml or 50µg/ml CBZ, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of TAC1was analysed using qPCR, standardised against the house keeping gene Pol II. Mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: ** = $P = < 0.01$.(n=3).

5.4.3 Modulation of NRSF & sNRSF localisation in human SH-SY5Y cells following ACD treatment.

5.4.3.1 Localisation of NRSF & sNRSF in human SH-SY5Y cells.

The localisation of a protein within the cell can play a key role in governing its function, and this is of particular relevance when regarding TFs. Previous work from our lab, and others, has shown that NRSF in predominantly localised within the cytoplasm in untreated neuronal cells (Zuccato *et al*., 2003; Spencer *et al*., 2006), whereas the truncated isoform is found to be nuclear (Magin *et al.,* 2002; Spencer *et al*., 2006). The

localisation of NRSF isoforms in our SH-SY5Y cells was consistent with these previous findings (Figure 5.7).

Antibodies previously used by our group to detect NRSF and the truncated isoform in the rodent (rREST4) (Spencer *et al*., 2006), were tested to determine if they detect the human NRSF isoforms, using SH-SY5Y protein extracts. The anti-NRSF (R2174) antibody detected a specific NRSF band at approximately 200kDa (Figure 5.7), which is consistent with findings from other groups (Ballas *et al.,* 2005b; Zuccato *et al*., 2003). The antibody raised to detect the truncated isoform (R3122), recognised a specific doublet at approximately 50kDa (Figure 5.6), consistent with other reports for the human isoform (Magin *et al*., 2002, Howard *et al*., 2008).

Using confocal microscopy and immunofluorescence, the localisation of NRSF in untreated human SH-SY5Y neuroblastoma cells was determined to be predominantly cytoplasmic, with some nuclear aggregations observed (Figure 5.7), consistent with previous findings in resting neurons (Zuccato *et al*., 2003; Spencer *et al*., 2006). In contrast, the truncated isoform was found to be predominantly nuclear, with strong overlay with the DAPI (Figure 5.7). Some cytoplasmic staining for the truncated isoform was also detected, matching previous observations by our group and others (Magin *et al.,* 2002; Spencer *et al*., 2006).

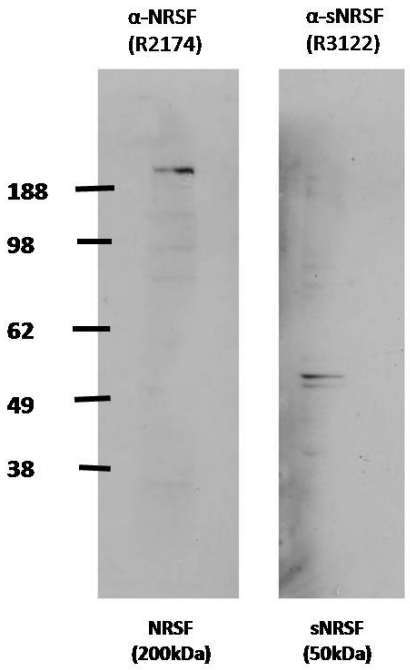

Figure 5.6 Western blotting of human SH-SY5Y cells extract with NRSF and sNRSF antibodies. Human SH-SY5Y cell extract probed with the anti-NRSF antibody (R2174) and anti-sNRSF (R3122). Specific NRSF antibody binding is observed at 200kDa and a doublet is observed with the anti-sNRSF at 50kDa.

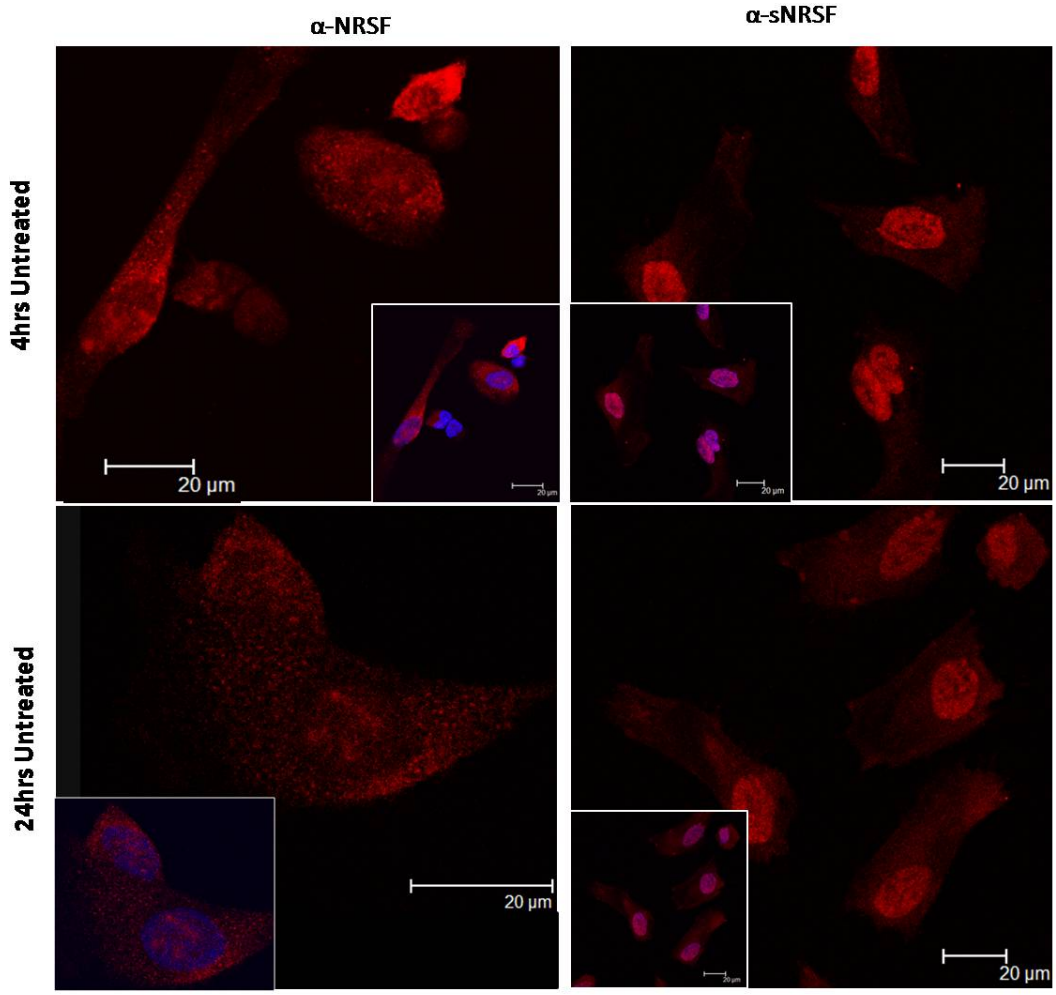

Figure 5.7 Immunofluorescence staining of NRSF isoforms in human SH-SY5Y cells. Untreated Human SH-SY5Y cells were stained with the anti-NRSF antibody (R2174) and anti-sNRSF (R3122).Both primary antibodies were recognized with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. NRSF is shown to be predominantly cytoplasmic, whilst the truncated isoform exhibits a more defined nuclear localisation, with some cytoplasmic staining observed. (n=2).

5.4.3.2 CBZ treatment and NRSF isoform localisation in SH-SY5Y cells.

It is known that KA treatment modulates the localisation of NRSF isoforms in rodent hippocampi (Spencer *et al*., 2006). Consequently, I was interested in exploring the role of each of the three ACDs used so far, if any, on NRSF isoform localisation in the human neuroblastoma SH-SY5Y cell line. NRSF localisation was found to be modulated by the vehicle control (Control C), with both 4hrs and 24hrs vehicle control cells, exhibiting a predominantly nuclear NRSF staining pattern, based on overlap with DAPI (Figure 5.8) (n=2). This nuclear staining pattern was also found following 4hrs and 24hrs 50µg/ml CBZ, indicating that CBZ treatment has no further affect upon NRSF localisation, compared to the vehicle control.

The vehicle control had little impact on the localisation of the truncated isoform, with well-defined nuclear staining plus some cytoplasmic staining, observed in (4hrs and 24hrs) vehicle control treated cells (Figure 5.9) (n=2), matching that observed in untreated cells (Figure 5.7) (n=2). The cytoplasmic staining of the truncated isoform was observed to be reduced following 24hrs 50µg/ml CBZ treatment, when compared to (24hrs) vehicle control treated cells (Figure 5.9) (n=2). However, this reduction has yet to be quantified explicitly, and so such reductions in staining are merely suggestive rather than conclusive. In addition, overall staining of the truncated isoform was found to be reduced compared to Control C (Figure 5.9) (n=2), which whilst not quantitative, is suggestive of a reduction in sNRSF levels, which would be consistent with that observed of mRNA levels following the same treatment (Figure 5.2b) (n=2). Analysis of protein levels would thus be of future interest.

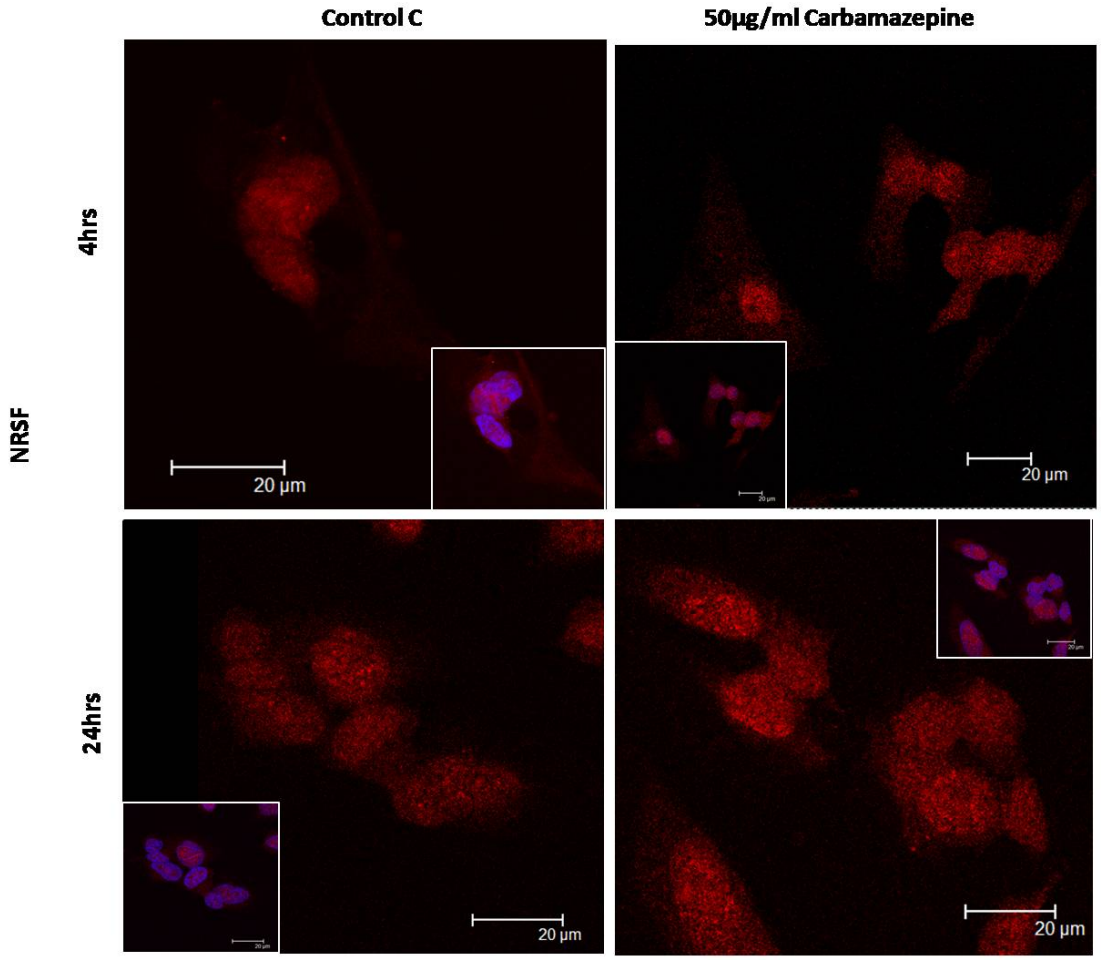

Figure 5.8 Immunofluorescence staining of NRSF in response to CBZ treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml CBZ or vehicle control (Control C). Cells were stained with the anti-NRSF antibody (R2174) and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. NRSF is shown to be nuclear based on overlay with DAPI, in Control C and CBZ treated cells. (n=2).

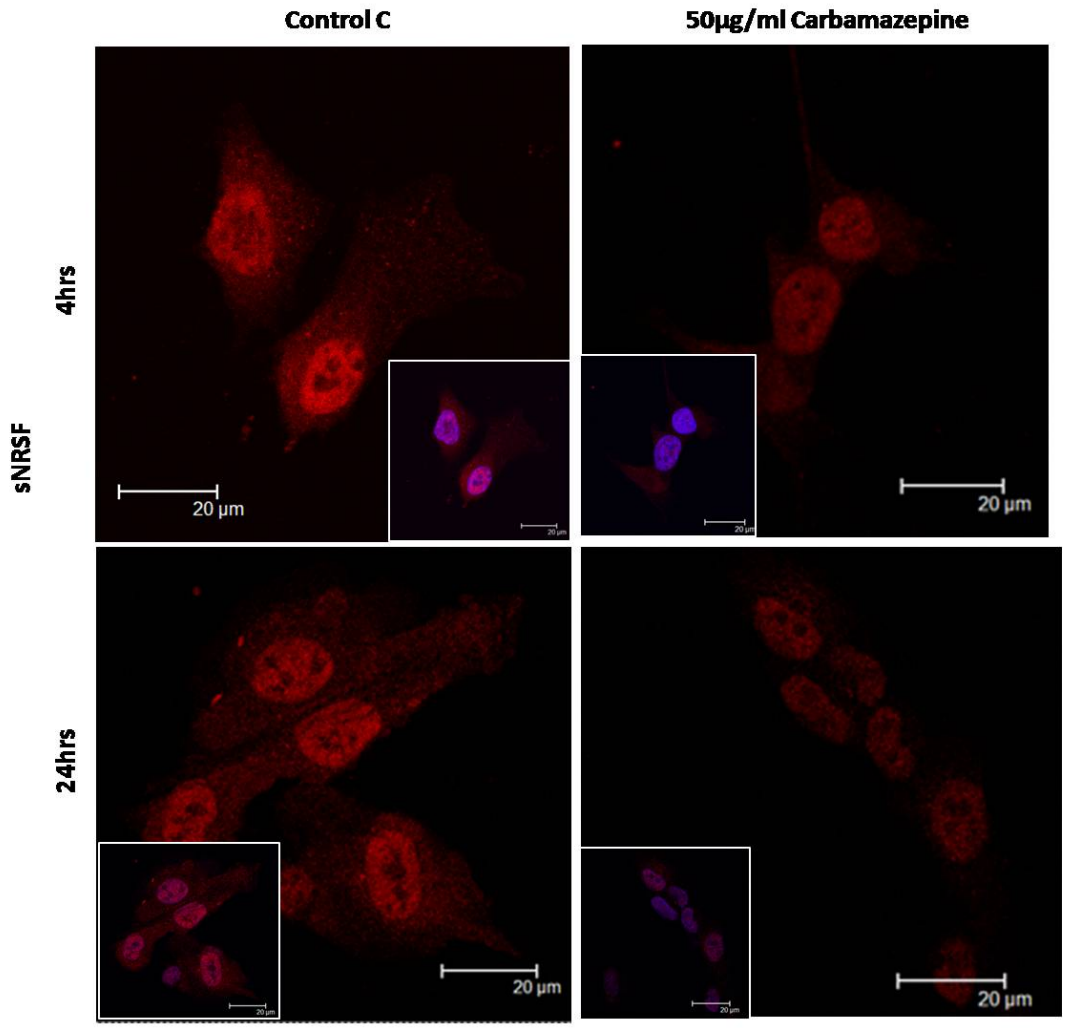

Figure 5.9 Immunofluorescence staining of the truncated isoform sNRSF in response to CBZ treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml CBZ or vehicle control (Control C). Cells were stained with the anti-sNRSF antibody (R3122) and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. sNRSF shows distinct nuclear staining in both Control C and CBZ treated cells, with reduced cytoplasmic staining following CBZ treatment observed. (n=2).

5.4.3.3 PHY and LMT treatment and NRSF isoform localisation in SH-SY5Y cells.

CBZ treatment was found to have a limited impact on the localisation of the NRSF isoforms, with only 24hrs 50µg/ml CBZ found to affect sNRSF localisation, with suggested reduced cytoplasmic staining. Both PHY and LMT had, in part, similarities to CBZ treatment in terms of modulating NRSF and sNRSF mRNA expression. Therefore I wanted to determine the impact of both of these ACDs on NRSF isoform localisation in human SH-SY5Y cells.

NRSF localisation in vehicle control (for PHY treatment) treated cells (Control P), was found to exhibit both a nuclear and cytoplasmic staining pattern (Figure 5.10) (n=2). The proportion of nuclear NRSF staining appeared greater in vehicle control (Control P) treated cells, , compared to untreated SH-SY5Y cells, however this is not quantified. Cells treated with 24hrs 50µg/ml PHY also exhibited nuclear NRSF localisation, with some cytoplasmic staining (Figure 5.10) (n=2), indicating that PHY, like CBZ, has no further impact upon NRSF localisation compared to its corresponding vehicle control. In contrast, the vehicle control (Control P) had no impact upon the staining pattern of the truncated isoform, compared to untreated SH-SY5Y cells, with the truncated isoform exhibiting a well-defined nuclear localisation, with some cytoplasmic staining also observed (Figure 5.11) (n=2). Similar to 24hrs 50µg/ml CBZ treatment, 24hrs 50µg/ml PHY treatment led to an apparent reduction in cytoplasmic staining of the truncated isoform, with virtually 100% nuclear staining, when compared against 24hrs vehicle controls (Control P) (Figure 5.11) (n=2). However, again this is not quantitative, and future analysis to measure the proportion of nuclear to cytoplasmic staining would be of interest.

Like CBZ and PHY, LMT treatment was found to have little impact upon NRSF localisation when compared to vehicle control treated cells, but did impact upon the cytoplasmic staining of the truncated isoform. NRSF localisation was found to be predominantly cytoplasmic with some nuclear aggregations observed, in (4hrs and 24hrs) vehicle control (Control L) treated cells (Figure 5.12) (n=2), matching that observed in untreated cells. LMT had no impact on this localisation pattern, with both 4hrs and 24hrs 50µg/ml LMT resulting in cytoplasmic NRSF localisation (Figure 5.12) (n=2). The truncated NRSF isoform was found to exhibit a well-defined nuclear localisation, with some cytoplasmic staining observed in vehicle control treated cells. 24hrs 50µg/ml LMT treated was found to reduced the cytoplasmic staining of the truncated NRSF isoform, compared to (24hrs) vehicle controls (Control L) (Figure 5.12) (n=2), whilst 4hrs LMT had little impact.

All three ACDs therefore had limited impact on the localisation of the NRSF isoforms, when compared to their corresponding vehicle controls. A general trend was observed, with the localisation of the full-length NRSF unaffected by the ACD treatment, whereas the cytoplasmic staining of the truncated isoform was reduced, following 24hrs treatment with CBZ, PHY or LMT, compared to vehicle control treated cells.

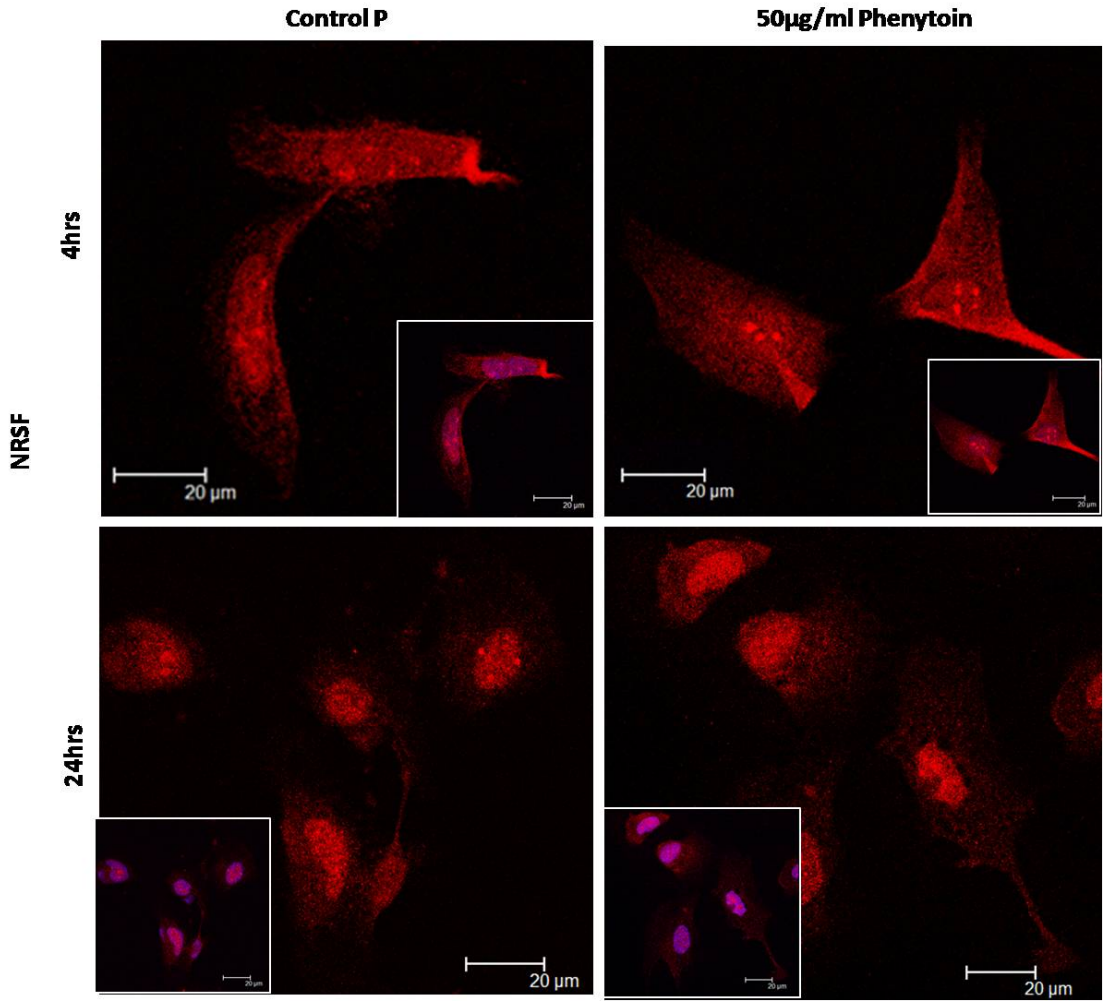

Figure 5.10 Immunofluorescence staining of NRSF in response to PHY treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml PHY or vehicle control (Control P). Cells were stained with the anti-NRSF antibody (R2174) and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. NRSF is shown to be nuclear, with some cytoplasmic and some nuclear aggregations in both Control P and PHY treated cells. (n=2).

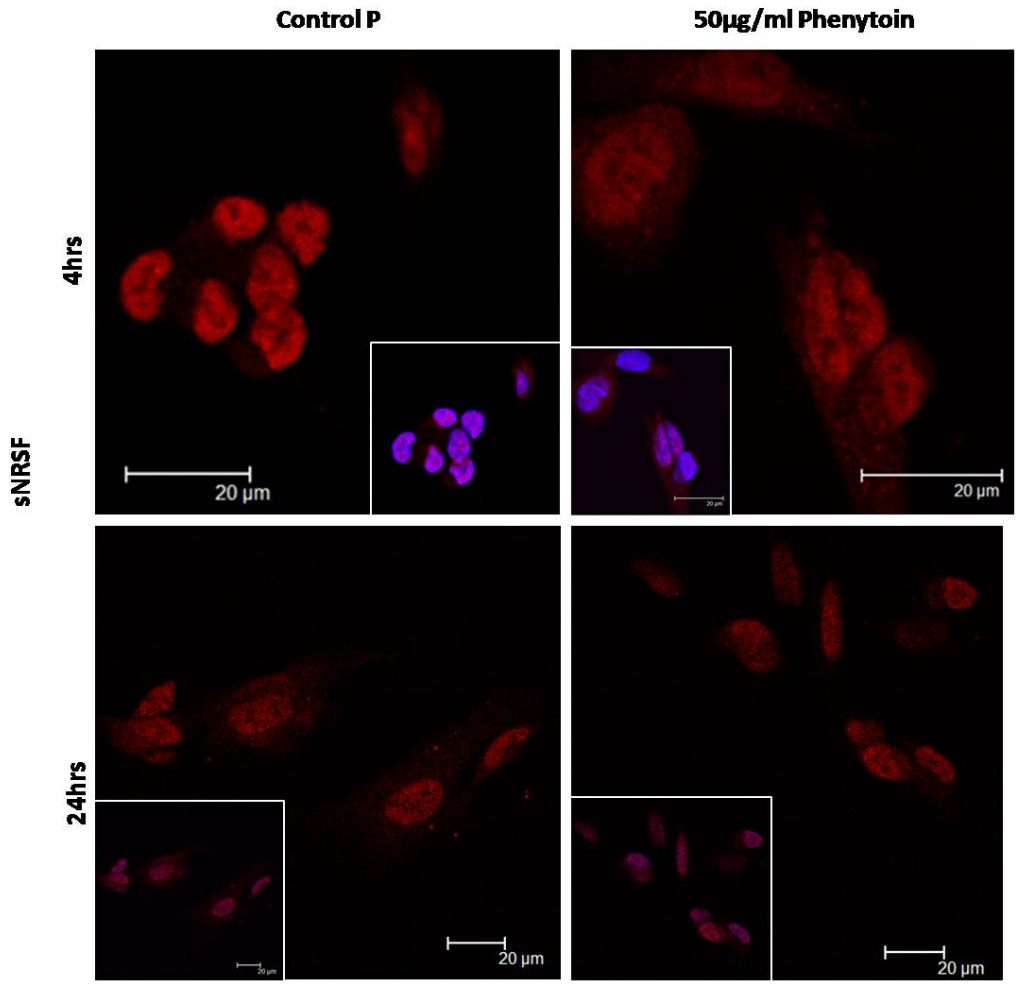

Figure 5.11 Immunofluorescence staining of the truncated isoform sNRSF in response to PHY treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml PHY or vehicle control (Control P). Cells were stained with the anti-sNRSF antibody (R3122) and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. sNRSF shows distinct nuclear staining in both Control P and PHY treated cells, with reduced cytoplasmic staining following 24hrs PHY treatment. (n=2).

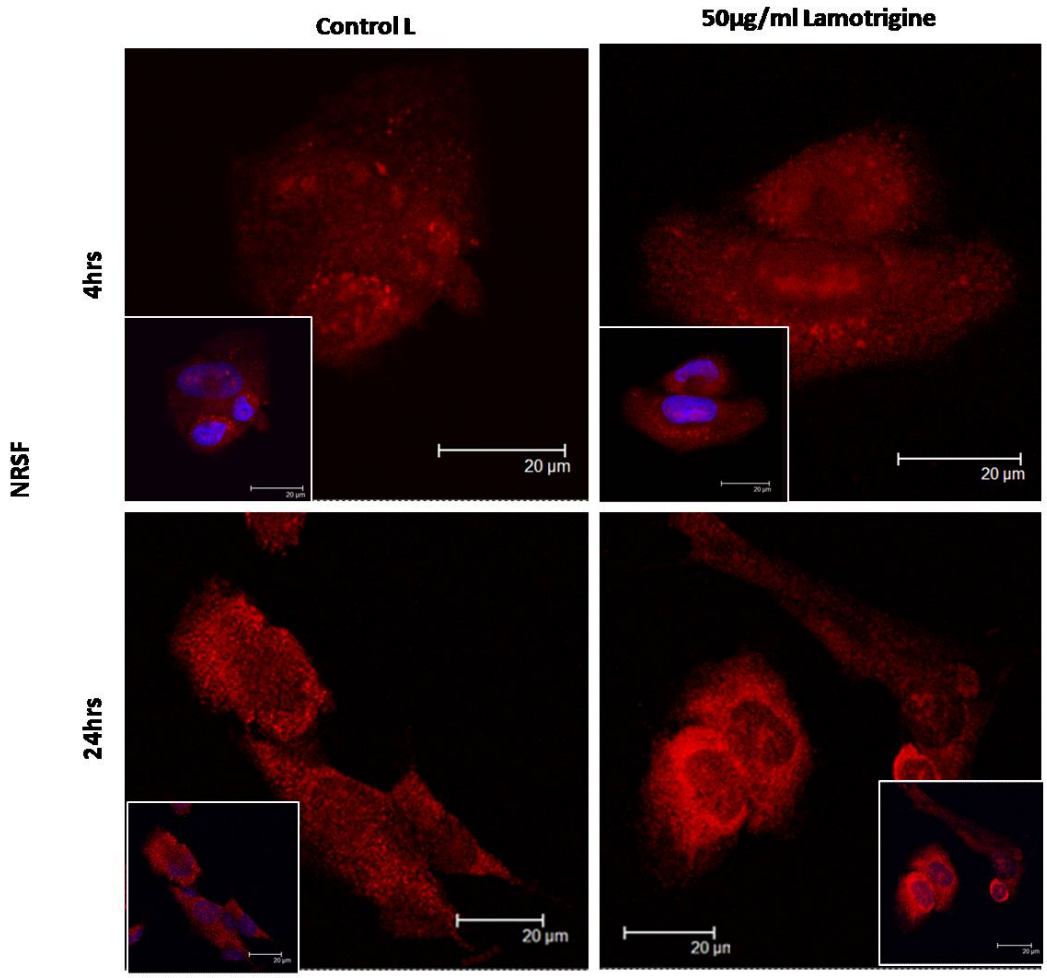

Figure 5.12 Immunofluorescence staining of NRSF in response to LMT treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml LMT or vehicle control (Control L). Cells were stained with the anti-NRSF antibody (R2174) and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. NRSF is shown to be non-nuclear, with cytoplasmic staining. (n=2).

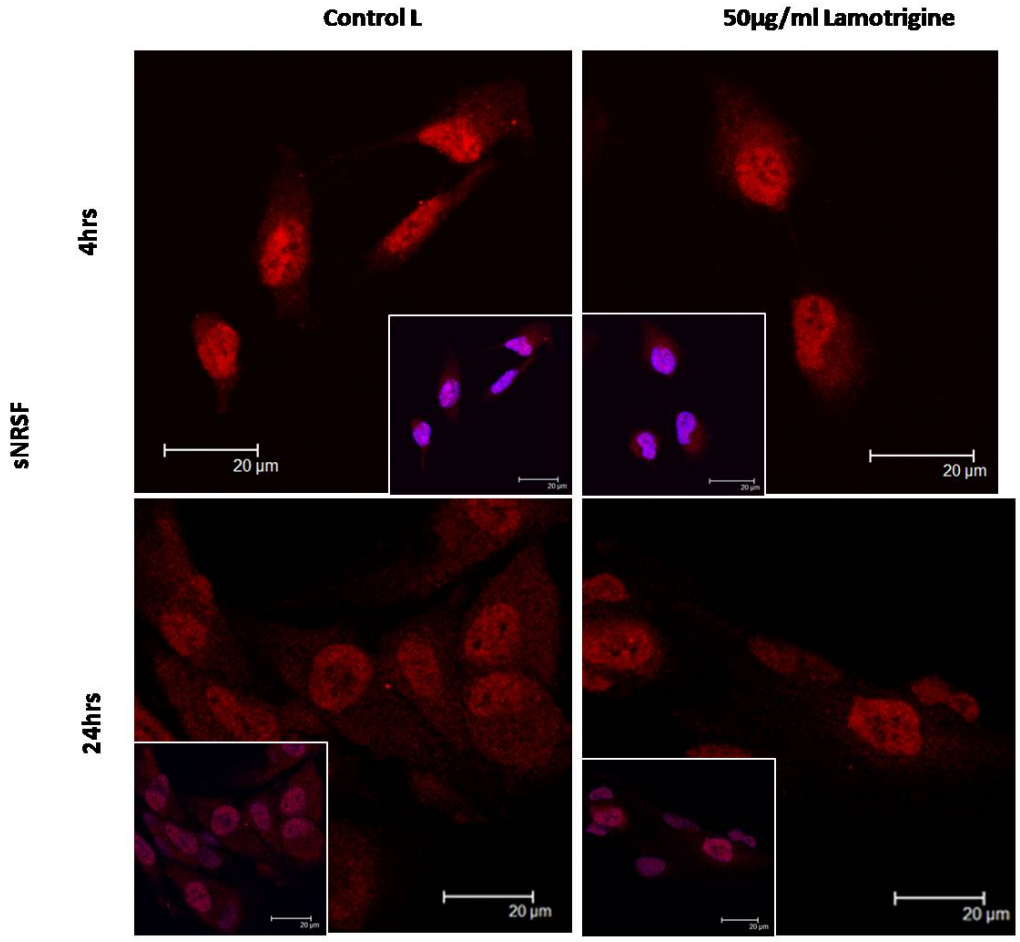

Figure 5.13 Immunofluorescence staining of the truncated isoform sNRSF in response to LMT treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml LMT or vehicle control (Control L). Cells were stained with the anti-sNRSF antibody (R3122) and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. sNRSF shows distinct nuclear staining in both Control L and LMT treated cells, with reduced cytoplasmic staining following 24hrs LMT treatment compared to Control L. (n=2).

5.4.3 Modulation of NRSF binding to target NRSE-containing regions in human SH-SY5Y cells by CBZ and PHY.

5.4.3.1 NRSF binding to target NRSE-containing regions in human SH-SY5Y cells.

An important aspect in NRSF mediated gene regulation is the ability of NRSF to recognise and bind to its 21bp consensus sequence, the NRSE. I was interested in exploring the possibility of ACD treatment affecting NRSF binding to target regions which contain the characterised NRSE of several neuronal genes. In the first instance, the binding of NRSF to a number of targets was explored to validate the ChIP assay.

To screen NRSF binding to target NRSE-containing regions, I opted to investigate a number of NRSF targets, including the 'classic' target genes SCG10 (Schoenharr & Anderson., 1995) and L1CAM (Kallunki *et al*., 1997), the neurotrophic factor BDNF (Timmusk *et al*., 1999), and the neuropeptides CART (Li *et al.,* 2008), TAC1 (Greco *et al*., 2007) and TAC3/NKB (Chapter 3). Each of these genes has a well characterised NRSE, with the exception of NKB, which has a putative NRSE as discussed in Chapter 3. A preliminary ChIP assay indicated that NRSF was able to bind to the NRSE-containing region of all 6 target genes, when compared to IgG background (negative control), in the human neuroblastoma cell line. NRSF binding was shown to be specific to the NRSE-containing regions, as no binding was observed using the GAPDH negative control PCR primer set (Figure 5.14) (n=1).

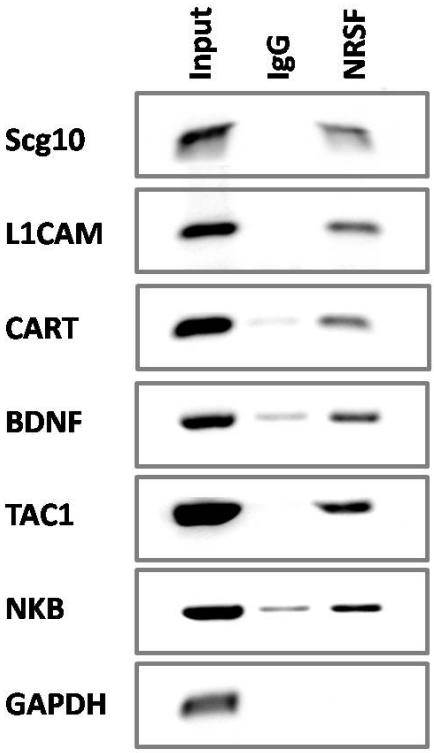

Figure 5.14. ChIP assay of NRSF binding to NRSE-containing regions in human SH-SY5Y cells. ChIP analysis of NRSF binding to characterised NRSE-containing regions in the genes Scg10, L1CAM, CART, BDNF, TAC1 and NKB (as described in chapter 3).IgG was included as a non-specific binding control (background). NRSF was found to bind to all characterised and putative NRSE-containing regions in untreated SH-SY5Y cells. Binding is shown to be specific to the NRSE locus of each gene, as no binding is observed in the GAPDH PCR negative controls.

5.4.3.2 NRSF binding to target NRSE-containing regions is modulated by ACD treatment in human SH-SY5Y cells.

To explore ACD modulation of NRSF binding to target NRSE-containing regions, I opted to treat cells with two ACDs, which differentially modulated NRSF and sNRSF mRNA expression levels, following 24hrs treatment: CBZ and PHY. As earlier discussed, 24hrs 50µg/ml CBZ treatment resulted in a significant decrease in mRNA

expression of both full-length NRSF and the truncated isoform sNRSF, compared to vehicle controls (Figure 5.2), whereas in contrast 24hrs 50µg/ml PHY resulted in a significant decrease in sNRSF mRNA only. Both of these drugs had no impact on NRSF localisation following 24hrs 50µg/ml treatment, but both did induced reduced cytoplasmic staining of the truncated isoform. Interestingly, the two drugs modulated NRSF binding to target regions, differentially, in a target specific manner.

Preliminary findings indicate that 24hrs 50µg/ml CBZ treatment led to increased NRSF binding to the SCG10, TAC1 and NKB (putative) NRSE-containing regions, compared to vehicle control (Control C) treated cells, (after standardising the NRSF pull-down PCR band intensity against the non-specific background control; IgG) (Figure 5.15). Interestingly, NRSF binding to the TAC1 and NKB promoter regions (which contain NRSEs) appears reduced in vehicle control treated cells, compared to untreated cells, whilst 50µg/ml CBZ enhances NRSF binding to these two regions, returning NRSF binding back to that observed in untreated cells (Figure 5.15). These findings suggest that the vehicle control has an impact on NRSF binding, however, it should be stressed that these are only preliminary findings, and replicate experiments are required before conclusions can be drawn. NRSF binding to the NRSE-containing regions of L1CAM, BDNF and CART was not different in vehicle control treated or CBZ treated cells, compared to untreated cell, thus suggesting that the modulation of NRSF binding could be target specific.

PHY treatment induced a different pattern of NRSF binding, compared to untreated and vehicle control treated cells. 24hrs 50µg/ml PHY resulted in a decrease in NRSF binding to the SCG10 NRSE-containing region when compared to both untreated

and vehicle control treated (Control P) cells (Figure 5.16). NRSF binding to the L1CAM, CART, TAC1 and NKB (putative) NRSE-containing regions was found to be reduced in vehicle control treated cells compared to untreated cells, whilst binding to the L1CAM and NKB (putative) NRSE-containing regions was restored back to untreated levels (Figure 5.16). In contrast 24hrs 50µg/ml PHY had no further impact upon the binding of NRSF to the CART and TAC1 NRSE-containing regions compared to vehicle controls. These findings may be supportive that ACDs can modulate NRSF binding to target regions, however as these findings are preliminary, repeated experimentation is required in the future.

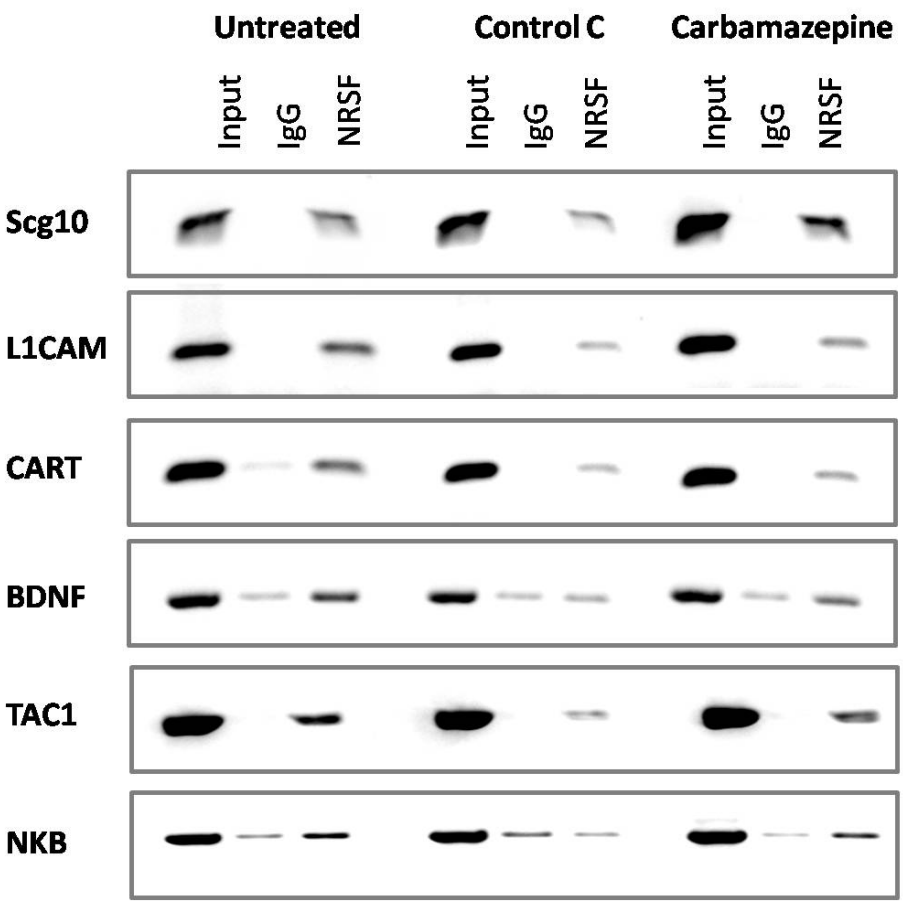

Figure 5.15. Modulation of NRSF binding to target NRSE-containing regions following CBZ treatment in human SH-SY5Y cells. ChIP analysis of NRSF binding to characterised or putative NRSE-containing regions in SH-SY5Y cells treated with either 24hrs 50µg/ml CBZ, vehicle control (Control C) or untreated. IgG was included as a non-specific binding control (background). NRSF binding to the Scg10, TAC1 and NKB NRSE-containing regions was found to be enhanced following CBZ treatment when compared to vehicle control. (n=1).

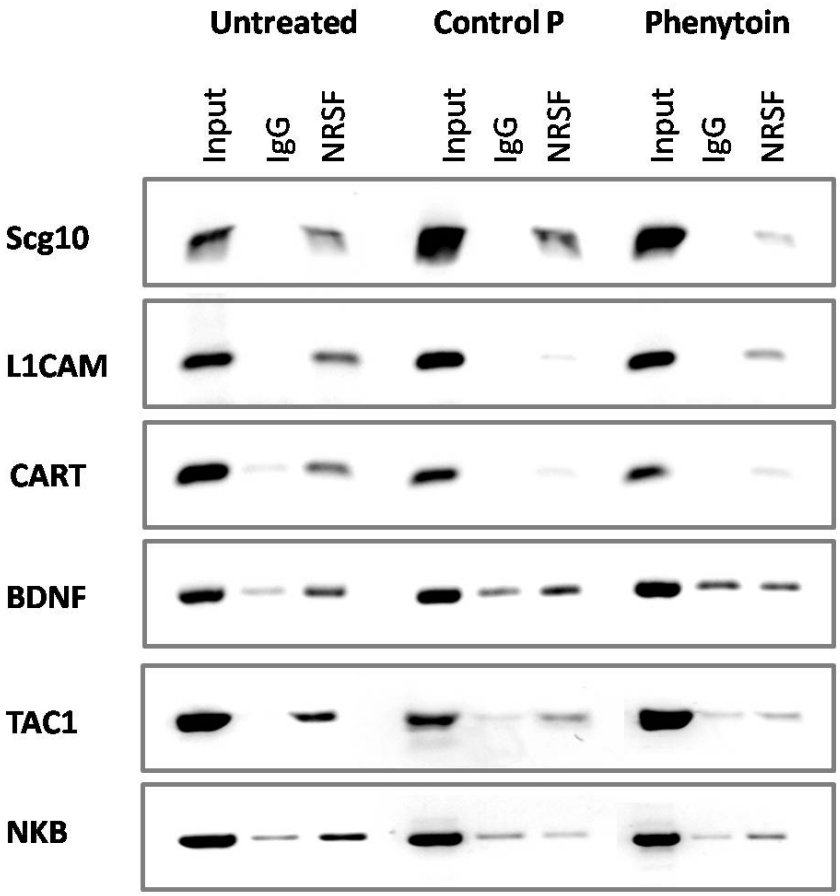

Figure 5.16. Modulation of NRSF binding to target NRSE-containing regions following PHY treatment in human SH-SY5Y cells. ChIP analysis of NRSF binding to characterised or putative NRSE-containing regions in SH-SY5Y cells treated with either 24hrs 50µg/ml PHY, vehicle control (Control P) or untreated. IgG was included as a non-specific binding control (background). NRSF binding to the Scg10 NRSE-containing locus was found to be reduced following PHY treatment when compared to vehicle control, whilst binding to the region encompassing the L1CAM NRSE appears enhanced. (n=1).

5.5 Discussion

The TF NRSF and its truncated isoform, have been shown to be key regulators of a number of neuronal genes implicated in epilepsy, including BDNF (Timmusk *et al*., 1999; Tabuchi *et al.,* 2002; Hara *et al*., 2009), TAC1 (Quinn *et al.,*2002; Spencer *et al.,* 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009; Chapter 4) and NKB (Chapter 3). In addition, both NRSF isoforms are known to be modulated in KA seizure models (Palm *et al*., 1999; Spencer *et al*., 2006), and NRSF has recently been shown to play a fundamental role in orchestrating the molecular response to the anti-epileptic ketogenic diet (Garrige-Canut *et al.,* 2006). Due to the apparent importance of NRSF mediated gene regulation in epilepsy, I sought to investigate the impact of ACD treatment on NRSF isoform expression, localisation and DNA binding in a human neuroblastoma cell line. Previous reports have suggested that the ACDs CBZ and LMT can induce elevated expression of BDNF in the rat cortex (Chang *et al,*2009), and I hypothesis this is due to the modulation of the NRSF regulatory system. Subsequently, the impact of these two ACDs, together with a third drug sharing a common sodium channel target, PHY (Kuo., 1998; Rogawski & Loscher., 2004a) upon NRSF isoform expression and localisation was explored.

5.5.1 NRSF isoform expression

NRSF isoform expression has been shown to be modulated by the pro-convulsant drug KA (Spencer *et al.,* 2006). In order to test the impact of ACD treatment, a cell line expressing both NRSF isoforms, which also exhibited NRSF modulation following KA stimulation, was required as an appropriate model. RT-PCR screening

revealed that the human neuroblastoma cell line SH-SY5Y expressed both full-length NRSF and the truncated isoform sNRSF, endogenously (see Chapter 3, Figure 3.1). In addition, both NRSF isoforms were found to be modulated following KA stimulation, with 24hrs (1μM and 5μM) KA inducing a significant increase in both full-length NRSF (Figure 5.1a) and the truncated isoform sNRSF (Figure 5.1b). Consequently, the human SH-SY5Y cell line was deemed to be an appropriate cell line to test the response of the NRSF TFs to ACD treatment.

Three ACDs were chosen for this study based upon their overlapping initial targets (sodium channel) and clinical uses, as previously discussed. Despite sharing the same initial target, each drug induced a different affect upon NRSF isoform expression. The1st generation ACD CBZ, was found to invoke differential modulation of both full-length NRSF and the truncated isoform sNRSF, in an exposure duration and drug concentration dependent manner. The shorter 4hrs exposure was found to induce a significant increase in NRSF expression (Figure 5.2a), whilst leading to a reduction in the expression of the truncated isoform (Figure 5.2b), whereas the longer 24hrs exposure, led to the significant reduction in both NRSF isoforms (Figure 5.2). CBZ is therefore a powerful modulator of NRSF isoform expression. To see whether CBZ treatment could also modulate expression of the NRSF regulated tachykinin gene, TAC1, the TAC1-expressing neuroblastoma cell line, SK-N-AS, was treated with CBZ. At all concentrations and exposure durations tested, TAC1 expression was significantly reduced (Figure 5.5). Thus, CBZ invokes a reduction in a pro-convulsant neuropeptide, which would be consistent with its function as an anti-convulsant, with this data providing evidence for a new mechanism of action for this ACD.

An alternative 1st generation ACDs, PHY, was found to have no impact on NRSF expression in the SH-SY5Y cell line, but did modulate the expression of the truncated isoform, sNRSF, with sNRSF significantly reduced following both 4hrs and 24hrs PHY treatment (Figure 5.3). Previous data published by our group, and others, and data presented in this thesis, implicates the truncated NRSF isoform as the more important NRSF isoform in epilepsy models. Our group has shown that the truncated isoform in more dynamically regulated in *in vivo* rodent seizure models, and drives activity of the rat TAC1 promoter to a greater extent than the full-length isoform (Spencer *et al.,* 2006), whilst in Chapter 4, only the over-expression of the HZ4 construct (expressing a truncated NRSF variant), and not the full-length NRSF construct (REEX1) led to further activation of the wild-type rat TAC1 promoter in cooperation with the USF proteins. Furthermore, the truncated isoform has also been shown to antagonise NRSF repression of the BDNF promoter (Tabuchi *et al*., 2002), thus implicating it in the regulation of BDNF, a key epilepsy associated gene (Koyama & Ikegaya., 2005). Due to the apparent importance of the truncated isoform in seizure-associated gene regulation, it is of particular interest that PHY treatment reduces the expression of the truncated isoform exclusively, without affecting the levels of full length NRSF. In addition, as PHY targets only the truncated isoform, this may be a useful drug in other conditions correlated with sNRSF over-expression, such as small cell lung cancer (Coulson *et al*., 2000).

LMT is a member of the 2nd generation of ACDs, which works similarly to CBZ and PHY, in terms of targeting voltage gated sodium channels (Kuo*.,* 1998), but has a more simplistic structure, and has reported fewer side effects due and lower patient

withdrawal from treatment, compared to both CBZ (Brodie *et al.,*1995) and PHY (Steiner *et al.,* 1999). LMT treatment was found to induce an increase in NRSF expression following 4hrs treatment, similar to CBZ, whilst having no impact following a more prolonged 24hrs treatment period (Figure 5.4a). This may suggest that LMT and CBZ work via a similar pathway, to modulate NRSF expression. The fact that both CBZ and LMT have previously been shown to modulate BDNF levels in the rat cortex (Chang *et al*., 2009) may be supportive of this theory. However, unlike the more prolonged 24hrs CBZ treatment, LMT treatment was not found to reduce NRSF expression. This may be due to differences in the stability or half life of LMT, within the cell, compared to CBZ. Certainly, the two drugs are known to be removed via different mechanisms, with CBZ removed through oxidation, governed by the cytochrome p450 gene CYP3A4 (Perucca., 2006; Pearce *et al.,*2008), whereas LMT is removed via a glucuronide conjugation controlled by the enzyme glucuronyl transferase type 1A4 (Magdalou *et al.,* 1992; Perucca., 2006). Due to these different routes of elimination, it is plausible that LMT may modulate (reduce) NRSF levels at different time points or treatment durations not tested here, and the only way to address this would be to repeat the experiment with different time courses.

Interestingly, LMT had no impact on the expression of the truncated isoform sNRSF, at the time points/treatment duration tested here. Again, differences observed between the three drugs, or more specifically LMT versus CBZ and PHY, in terms of sNRSF modulation, may be due to differences in the half-life of these three drugs. As with CBZ, PHY is eliminated through oxidation, this time orchestrated by CYP2C9 and CYP2C19 (Perucca., 2006; Mosher *et al*., 2009). Perhaps the lack of sNRSF modulation

by LMT is due to its route of elimination, which may be quicker or longer than that of CBZ and PHY, and perhaps one is missing any sNRSF modulation induced by LMT at these 4hrs and 24hrs time points. As mentioned, one would have to repeat the treatment at different, perhaps shorter time points, to conclude if LMT does have an impact on sNRSF expression.

5.5.2 NRSF isoform localisation

The localisation of a protein within the cell can play a key role in governing its function, and this is of particular relevance when regarding TFs. Previous work from our lab, and others, has shown that NRSF in predominantly localised within the cytoplasm in untreated neuronal cells (Zuccato *et al*., 2003; Spencer *et al*., 2006), whereas the truncated version is found to be nuclear (Spencer *et al*., 2006). The localisation of NRSF and its truncated isoform in SH-SY5Y cells was found to be consistent with these previous reports. The localisation of NRSF is known to be controlled by two distinct proteins: Huntingtin (Zuccato *et al*., 2003) and the RILP (Shimojo & Hersh, 2003). Huntingtin has been shown to sequester NRSF in the cytoplasm, with the mutant Huntingtin protein (the hallmark of HD) failing to sequester NRSF into the cytoplasm, leading to nuclear accumulation of NRSF (Zuccato *et al*., 2003). In contrast, RILP is known to govern the nuclear localisation of NRSF, with cytoplasmic localisation of NRSF found upon knock-down of RILP (Shimojo Hersh., 2003).

NRSF localisation is of particular interest when regarding seizure, as the localisation of the NRSF isoforms switches following pro-convulsant KA treatment (Spencer *et al*., 2006). More recently, a mutation in the RILP gene was found to cause

an autosomal-recessive PME-Ataxia syndrome (Bassuk *et al*., 2008). Taken together, the localisation of NRSF isoforms appears to be an important aspect in seizure. Subsequently, I sought to explore the potential affect of ACD treatment on the localisation of NRSF and sNRSF in human SH-SY5Y cells.

ACD treatment was found to have little impact upon the localisation of full-length NRSF, following the treatments tested here. The ACDs did however all lead to an apparent reduction in cytoplasmic staining of the truncated isoform, following 24hrs treatment, which may be supportive of the greater importance of the truncated isoform in seizure, as the ACDs only had an impact upon the truncated isoform's sub-cellular distribution. However, it is important to state that this data is not quantitative, and whilst the staining patterns observed suggests a reduction in cytoplasmic staining of the truncated isoform occurs, a more accurate approach is required in future investigations. For example, one could analyse the protein levels of the truncated isoform in both cytoplasmic and nuclear extracts, via a western blot, to determine if the change in staining observed, is a true reflection on a change in protein levels in the cytoplasm. Interestingly, the vehicle controls for CBZ treatment and PHY treatment had a marked affect upon NRSF localisation, resulting in a more apparent nuclear localisation patterns compared to untreated cells. Therefore it is evident that the vehicle itself induces a shift in NRSF localisation, which may be masking any affect CBZ or PHY have. It would therefore be of interest to repeat the experiments with different vehicles, for example, CBZ could be prepared in propylene glycol instead of ethanol.

The fact that NRSF localisation was unaffected by ACD treatment compared to vehicle controls, and that the localisation of the truncated isoform was only moderately

affected, may well be consistent with the roles of these drugs in restoring neuronal cells back from a hyper-excitable state, to a more normal state. The fact that these drugs do not affect the localisation of NRSF isoforms, is perhaps a good thing, after all, it is during seizures or indeed in HD, that abnormal NRSF localisation is found.

It may be of interest to explore the role of these ACDs in modulating NRSF isoform localisation, following seizure conditions. For example, it would be particularly interesting to see if KA induced a change in NRSF isoform localisation within these cells, and if the ACD treatment could return the NRSF isoform localisation back to pre-KA or pre-seizure like conditions. Data from such an experiment may support a theory that these drugs only affect NRSF localisation, when abnormal localisation is observed, restoring it back to a normal state.

5.5.3 NRSF binding to target NRSE-containing regions

In addition to modulation of expression and localisation, the regulatory role of a TF can be affected by modulating its ability to recognise and bind to its recognition DNA motif. Previous studies have shown that NRSF binding its recognition motif, the NRSE, is modulated following ischemic insults, with NRSF binding to the μ-opioid receptor (MOR-1) gene promoter, enhanced following ischemia in the rat hippocampus (Formisano *et al*., 2007). Furthermore, in a mouse transgenic model of HD, NRSF binding to target NRSEs in the BDNF, SYN1, Chrm4, Drd3 and Chrmb2 genes, was found to be elevated in the HD mice compared to wild-type mice (Zuccato *et al*., 2007). To investigate whether ACD treatment may affect NRSF binding to target NRSE-containing regions, a preliminary ChIP assay was undertaken to explore the impact of

24hrs CBZ and 24hrs PHY treatment on NRSF binding to the regions containing the characterised or putative NRSE, of the genes SCG10, L1CAM, BDNF, CART, TAC1 and TAC3.

The preliminary findings suggested that both CBZ and PHY could affect NRSF binding to NRSE-containing regions.. Whilst neither treatment affected NRSF binding to the regions encompassing the BDNF or CART NRSEs, they did differentially modulate binding to SCG10, L1CAM, TAC1 and TAC3 NRSE-containing regions. CBZ treated resulted in elevated NRSF binding to the SCG10, TAC1 and NKB NRSE-containing regions compared to vehicle controls (Figure 5.15). Interestingly, NRSF binding to TAC1 and NKB promoter regions appeared reduced in vehicle control treated cells, when compared to untreated cells, suggesting that the vehicle control may be having an impact upon NRSF binding. . Similarly, the vehicle control for PHY treatment also induced a reduction in binding to regions encompassing the L1CAM, CART, TAC1 and NKB NRSEs, whilst PHY treatment appeared to restore NRSF binding to the L1CAM and NKB NRSE-containing regions (compared to vehicle control) (Figure 5.16). Whilst these findings are of interest, it should be stressed that they are preliminary findings, and it is therefore of particular interest to repeat these experiments in the future to gain a true reflection of any ACD-mediated modulation of NRSF binding to target regions.

One common trend noted following these two preliminary investigations was the suggestive elevated NRSF binding to the NKB promoter, compared to vehicle controls. This is an intriguing finding, as I show in chapter 3 that NRSF is important in NKB regulation, acting as an activator in human neuroblastoma cells, and thus one may

expect an ACD to reduce NRSF binding to the NKB promoter, which may lead to reduced NKB expression. However, multiple TFs were found to be important in NKB regulation, including USF proteins and the truncated NRSF variant, and thus binding of these factors to the NKB promoter region needs to be explored to better understand the affects of ACD treatment on NKB regulation.

Whilst these findings are preliminary, they do present interesting differences between the different ACDs which may be explained by their differential affects on the expression of full-length NRSF. Both CBZ and PHY led to a reduction in sNRSF mRNA expression, but only CBZ affected full-length NRSF expression, causing a reduction at the 24hrs time point tested here. Interestingly, despite a reduction in NRSF mRNA expression, NRSF binding to the regions containing the SCG10, TAC1 and NKB NRSEs, appeared to increase. One possible explanation for such differences could be due to the ratio of NRSF to sNRSF. It is predicted that the truncated isoform can bind, albeit weakly, to target NRSEs, and thus may compete or antagonise NRSF binding to target NRSEs (Tabuchi *et al*., 2002). Thus following PHY treatment, where NRSF expression is unaffected, but the truncated isoform is reduced; the ratio between the two isoforms is elevated in favour of full-length NRSF, which is in contrast to that observed following CBZ treatment, whereby both isoforms are reduced. Such differences in the ratio of NRSF:sNRSF, may help explain the observed differences in NRSE binding to target regions.

As stated earlier, binding of NRSF to the region encompassing the BDNF NRSE was unaffected by ACD treatment, in these preliminary experiments. Previous studies have also shown that NRSF binding to the BDNF NRSE is unaffected by the anti-

epileptic ketogenic diet, more specifically by glycolytic inhibition (Garriga-Canut *et al.,* 2006). However, the ketogenic diet model did induce a marked change in chromatin structure around the BDNF promoter, indicated by an increase in H3K9 methylation (marker of closed, repressed chromatin) and a decrease in H3K9 acetylation (marker of open, active chromatin) (Garriga-Canut *et al* 2006). It would therefore be of interest to extend future investigations to explore whether pro-convulsant or ACD treatments, affect chromatin markers, in the human neuroblastoma cell model employed here.

To summarise, ACD treatment targets and modulates the NRSF regulatory system, in a differential manner. Each drug elicited differential modulation of NRSF isoform mRNA expression at the concentrations and exposure durations tested here, whilst in general, maintaining and not disturbing NRSF isoform localisation. Finally, preliminary investigations suggest that ACD treatment may modulate NRSF binding to specific target regions. These findings provide new evidence for the function of these ACDs in modulating a distinct TF regulatory system, which is implicated in seizure progression and maintenance.

CHAPTER 6: USF1 and USF2 are modulated by ACD treatment

6.1 Introduction

In earlier chapters, a regulatory role for the USF TFs in governing both NKB (chapter 3) and TAC1 (chapter 4) expression was discussed. Both of these tachykinin genes are considered to be pro-convulsant (Marksteiner *et al*., 1992; Wasterlain *et al*., 2002; Chen *et al*., 2008; Liu *et al*, 1999; Wasterlain *et al*, 2000) and as such are important in seizure. A number of bHLH factors have been shown to be modulated following seizure *in vivo*, including Mash1, Id2 and Hes5 (Elliot *et al*., 2001), which suggests that the USF TFs may also be modulated following seizure. In support of this, USF knockout mice are known to be prone to spontaneous seizures (Sirito *et al.,* 1998), and the USF TFs are also known to regulate a host of neuronal genes implicated in epilepsy, including BDNF (Tabbuchi *et al*., 2002b), the $GABA_B$ receptor (Steiger *et al.,* 2004) and KCC2 (Markkanen *et al*., 2008). Despite these facts, the response of the USF family to seizure has not yet been explored. However, due to the proposed role of USFs in epilepsy, I sought to investigate whether USF expression, localisation and function (DNA binding) were affected by ACD treatment, as was tested in chapter 5 for the NRSF isoforms.

As discussed in chapter 5, the expression of the USF and NRSF-regulated gene BDNF has been shown to be up-regulated following VPA (Kim *et al*., 2007), CBZ and LMT (Chang *et al,* 2009) treatment, which may in part be due to the modulation of NRSF following such drug treatment (Kim *et al*., 2007; Chapter 5), but may also be due to these drugs modulating other TFs such as USF1 or USF2. Evidence to suggest that

ACDs may modulate USF1 or USF2 expression includes the observed down-regulation of the USF-regulated KCC2 gene, following VPA treatment in rat cortical neurons (Fukuchi *et al*., 2009). To determine if such drugs could modulate USF gene expression, the expression of both USF1 and USF2 was monitored following CBZ, PHY and LMT treatment. These three ACDs were employed in chapter 5 due to the fact that they can function via the same sodium channel blockade mechanisms, but can have different downstream targets, and different clinical use.

By addressing USF modulation following ACD treatment, it is hoped that molecular mechanisms which play pivotal roles in seizure generation and progression, may be unearthed. Furthermore, our knowledge about the mechanisms by which ACD exert their effects may well be enhanced by screening for USF modulation here, to supplement the findings described in chapter 5.

6.2 Aims

- To test if pro-convulsant treatment (KA) modulates the mRNA expression of the USF TFs in human neuroblastoma cells (SH-SY5Y).
- To explore the impact of ACD treatment on USF1 and USF2 mRNA expression in SH-SY5Y cells, using the clinically prescribed ACDs CBZ, PHY and LMT.
- To explore USF1 and USF2 localisation in human SH-SY5Y neuroblastoma cells.
- To determine if the ACDs affect USF localisation in SH-SY5Y cells.
- To determine USF binding to neuropeptide promoter regions in SH-SY5Y cells using ChIP.
- To explore the effects of ACD treatment on USF binding to target neuropeptide promoter regions.

6.3 Methods

6.3.1 Cell culture and treatment

Human SH-SY5Y cells were used throughout as RT-PCR revealed they express both USF1 and USF2 (see chapter 3, Figure 3.1). SH-SY5Y cells were cultured as outlined in methods section 2.2.2.1.1 and treated with KA, ACDs or vehicle controls as described in section 2.2.2.2. KA, ACDs and the corresponding vehicle controls were prepared as described in section 2.1.3.For mRNA analysis, cells were then harvested as detailed in methods section 2.2.3.1, 2.2.3.3 and measured as described in section 2.2.3.5. For immunofluorescence, cells were fixed and processed as described in methods section 2.2.4.5 and for ChIP assays, cells were fixed and protein/DNA interactions cross-linked as described in section 2.5.1.

6.3.2 mRNA expression analysis

The expression of the TFs USF1 and USF2, as well as the house keeping gene Pol II, was investigated using qPCR as described in methods section 2.2.3.5, using the PCR primers set out in Table 2.2.1. Thermal cycling conditions for USF 1, USF2 and Pol II, were as follows: initial denaturation: 95^{o}C for 3mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds and annealing 62^{o}C for 30 seconds; and a final elongation step of 72^{o}C for 2mins. Specificity of products was determined by subsequent melt curve analysis from 55°C to 95°C increasing in 0.5°C increments

6.3.3 Protein analysis

6.3.3.1 Western blotting

To test the anti-USF1 and anti-USF2 antibodies, total protein was extracted from untreated SH-SY5Y cells as described in section 2.2.4.1. Western blotting was performed as outlined in methods section 2.2.4.3.

6.3.3.2 Immunofluorescence

SH-SY5Y cells cultured on cover-slips as described in section 2.2.2.1.2, treated as in section 2.2.2.2, and harvested as described in section 2.2.4.4. Cover-slip mounted cells were probed with antibodies described in Table 2.3, and mounted and visualised as outlined in section 2.2.4.4.

6.3.4 TF binding study – ChIP assay

To investigate USF2 TF binding to the human TAC1 and NKB promoter region, ChIP was employed as outlined in method section 2.2.5, using the antibodies given in Table 2.3 and the PCR primers given in Table 2.4. USF1 binding was not explored as conditions could not be optimised in time for completion of this thesis.

6.4 Results

6.4.1 Modulation of USF1 and USF2 mRNA following KA treatment.

To explore the impact of ACD treatment on the expression of the USF TFs, a cell-line model was required which was responsive to pro-convulsant challenge. In Chapter 5, the human SH-SY5Y cell line was found to respond to KA, with marked up-regulation of the NRSF isoforms observed. The SH-SY5Y cell line was again used here due to this response to pro-convulsant stresses, but also as both USF1 and USF2 was found to be endogenously expressed (Figure 3.2). In addition, using the same cell line and treatment meant that comparisons between NRSF isoform and USF TF modulation could be made. To determine the response of USF1 and USF2 to pro-convulsant challenge, SH-SY5Y cells were treated with either 1μM or 5μM KA, or vehicle control, for either 4hrs or 24hrs, before being processed for RNA extraction. USF1 and USF2 mRNA levels were quantified via qPCR and standardised relative to Pol II.

Differential modulation of both USF1 and USF2 mRNA expression was observed following KA treatment. USF1 modulation was found to be concentration and exposure-duration dependent , with 4hrs 1μM KA inducing a marked 3x-fold increase ($P = < 0.01$), whilst 4hrs 5μM KA induced a less substantial 50% increase ($P = < 0.001$) (Figure 6.1a) (n=3). With the more prolonged 24hrs KA treatment, only the higher 5μM KA concentration elicited a change in USF1 mRNA expression, with a significant 2x fold increase noted ($P = < 0.01$) (n=3) (Figure 6.1a). qPCR also revealed that 24hrs 5μM KA induced a 4x fold increase in the expression of USF2 (Figure 6.1b)

(*P = < 0.05*) (n=3). To my knowledge, these are the first findings that USF TFs are up-regulated following KA treatment.

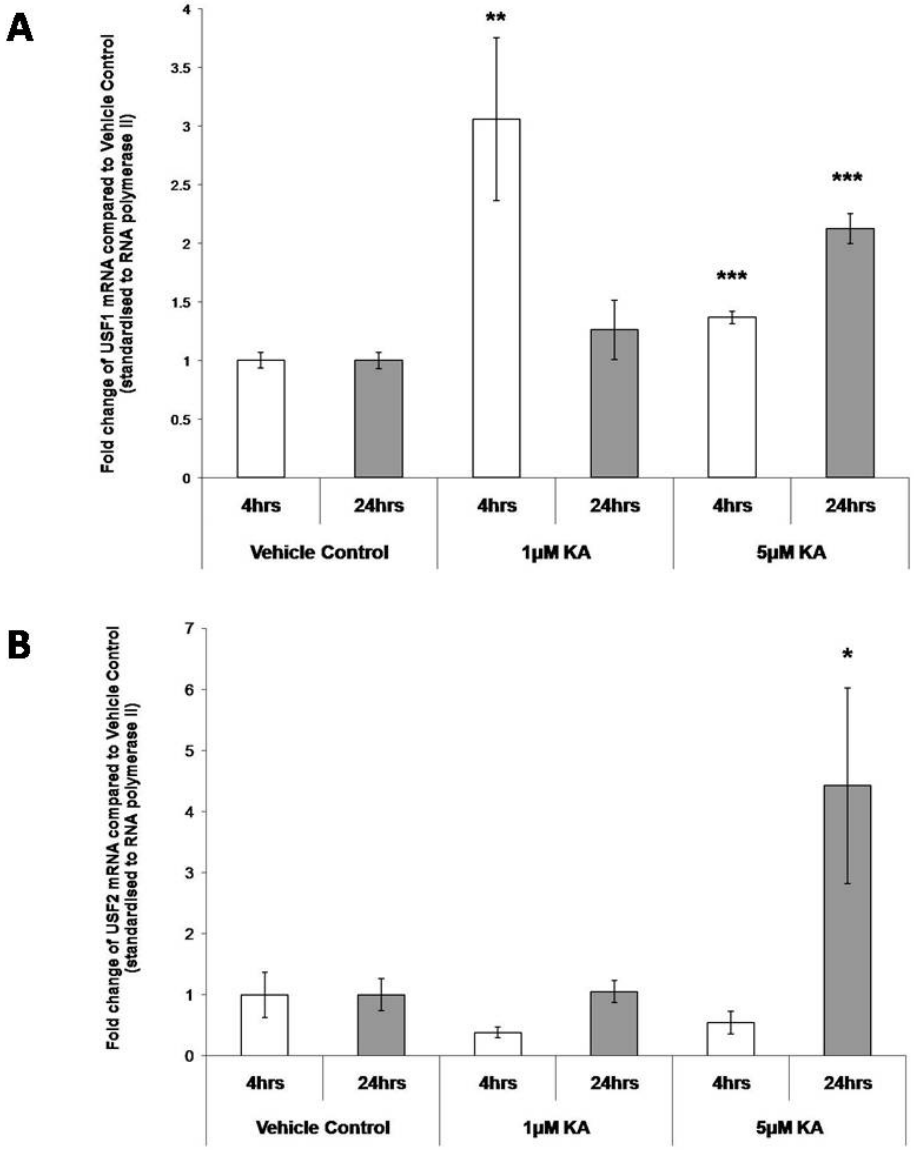

Figure 6.1 Modulation of the USF TFs by KA. qPCR analysis of **(A)** USF1 and **(B)** USF2 mRNA expression in response to KA treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 1μM or 5μM KA, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of USF1 and USF2 were analysed using qPCR, standardized against the house keeping gene Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = *P = < 0.05*; ** = *P = < 0.01* & *** = *P = < 0.001*. (n=3).

6.4.2 Modulation of USF1 and USF2 expression following ACD treatment.

In chapters 3 and 4, the importance of the USF TFs in the regulation of the pro-convulsant tachykinins NKB and TAC1 was highlighted, and USF proteins are known to regulate another epilepsy gene associated, BDNF (Tabbuchi *et al.,* 2002b*)*. In chapter 5, the NRSF isoforms, also implicated in NKB, TAC1 and BDNF regulation, were found to be modulated by ACD treatment. I was thus interested in extending this analysis to the USF TFs, by exploring the effect of ACD treatment on USF1 and USF2 mRNA expression. The three ACDs used in chapter 5, were again utilised here, those being CBZ, PHY and LMT. As previously discussed, CBZ, PHY and LMT are proposed to function via a similar mechanism, but despite this, differential modulation of the USF TFs expression was observed.

6.4.2.1 CBZ modulation of USF1 & USF2 mRNA

CBZ treatment was found to induce a time-dependent change in USF1 mRNA expression, with 4hrs 10µg/ml CBZ inducing a highly significant 75% reduction in USF1 mRNA, and 4hrs 50µg/ml CBZ inducing a 60-70% reduction (Figure 6.2a) ($P = < 0.001$) (n=3), when compared to vehicle control (Control C). In contrast, the more prolonged 24hrs exposure had no impact on USF1 mRNA levels (Figure 6.2) ($P = > 0.05$) (n=3).CBZ treatment also led to a repression of USF2. 4hrs 10µg/ml and 50µg/ml CBZ treatment induced a significant 60% reduction in USF2 mRNA expression ($P = < 0.01$) (n=3), whilst the more prolonged 24hrs 10µg/ml and 50µg/ml CBZ treatment led to a even more significant decrease (70-80%) ($P = < 0.001$) (n=3) (Figure 6.2b).

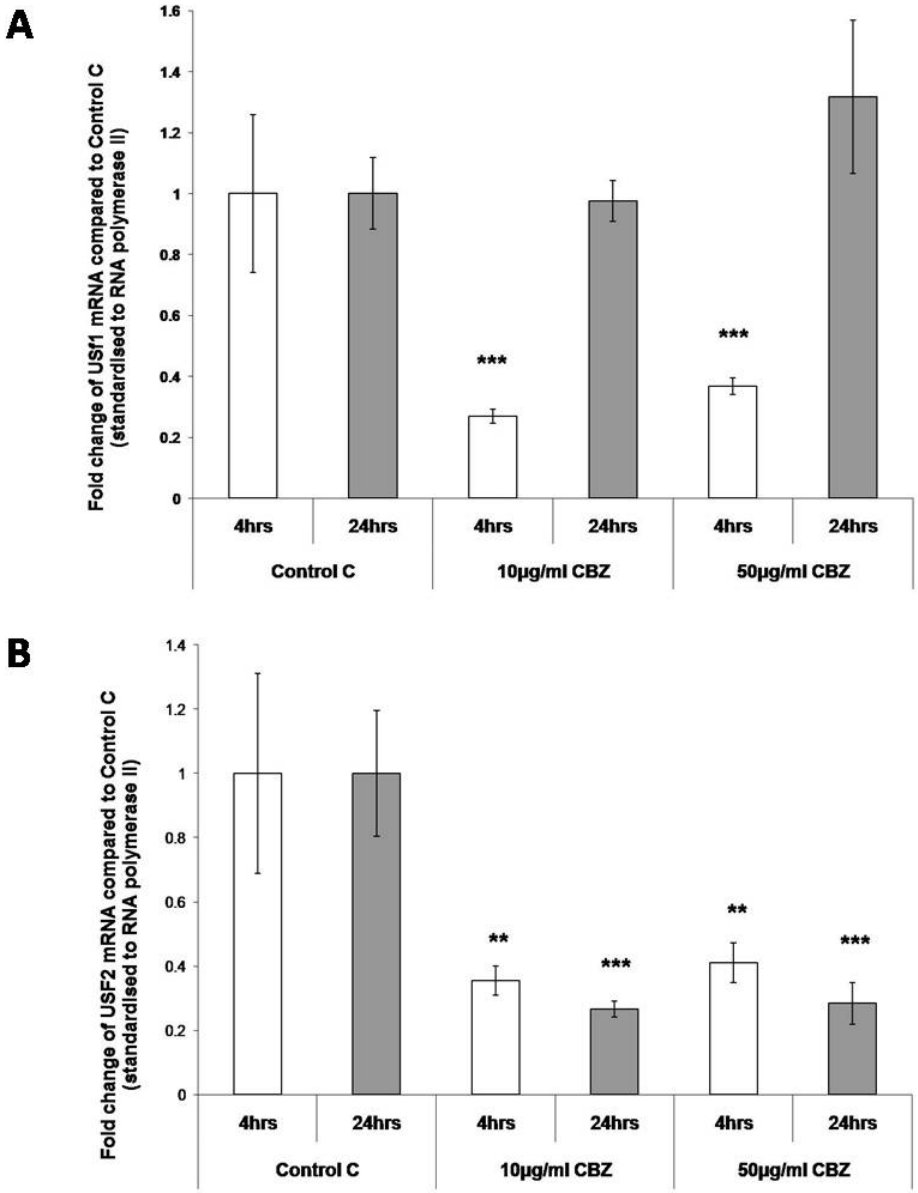

Figure 6.2 Modulation of the USF TFs by CBZ. qPCR analysis of **(A)** USF1 and **(B)** USF2 mRNA expression in response to CBZ treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 10µg/ml or 50µg/ml CBZ, or vehicle control (Control C), for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of USF1 and USF2 were analysed using qPCR, standardized against the house keeping gene Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: ** = $P = < 0.01$ & *** = $P = < 0.001$. (n=3).

6.4.2.2 PHY modulation of USF1 & USF2 mRNA

In chapter 5, PHY was found to differentially modulate NRSF isoform expression, when compared to CBZ, despite sharing the same initial target (kuo *et al.,* 1998). Here, PHY is again found to elicit a different response to CBZ in terms of USF1 and USF2 mRNA expression. PHY modulation of USF1 was found to be concentration and exposure-duration dependent, with only 4hrs 10µg/ml PHY resulting in a significant 2x fold increase in USF1 expression ($P < 0.05$) (n=3) (Figure 6.3a). PHY treatment had no significant impact upon USF2 expression ($P = > 0.05$) (n=3) (Figure 6.3b).

6.4.2.3 LMT modulation of USF1 & USF2 mRNA

Previous reports have shown that both CBZ and LMT can modulate BDNF expression in the rat cortex (Chang *et al,*2009), and in chapter 5 I proposed that this is due to similar affects of these drugs on the NRSF regulatory system. BDNF is also known to be regulated by USF proteins (Tabbuchi *et al.,* 2002b), and thus it was of interest to see if CBZ and LMT could affect the USF regulatory system, as an alternative explanation for this reported change in BDNF expression. Here, LMT was found to modulate USF1 and USF2 expression, in a manner similar to CBZ, mirroring the similar affect of LMT to CBZ treatment upon NRSF expression (see chapter 5). Both 4hrs 10µg/ml and 50µg/ml LMT led to a highly significant 80% reduction in USF1 mRNA expression, when compared to vehicle controls (Control L) (Figure 6.4a) ($P = < 0.001$) (n=3), whilst the prolonged 24hrs LMT treatment had nil affect ($P = > 0.05$). This modulation was found to be mirrored with USF2 expression, with both 4hrs 10µg/ml

and 50µg/ml LMT inducing a highly significant 60% reduction in USF2 (Figure 6.4b) (*P = < 0.001*) (n=3), whilst 24hrs had no impact, compared to vehicle controls.

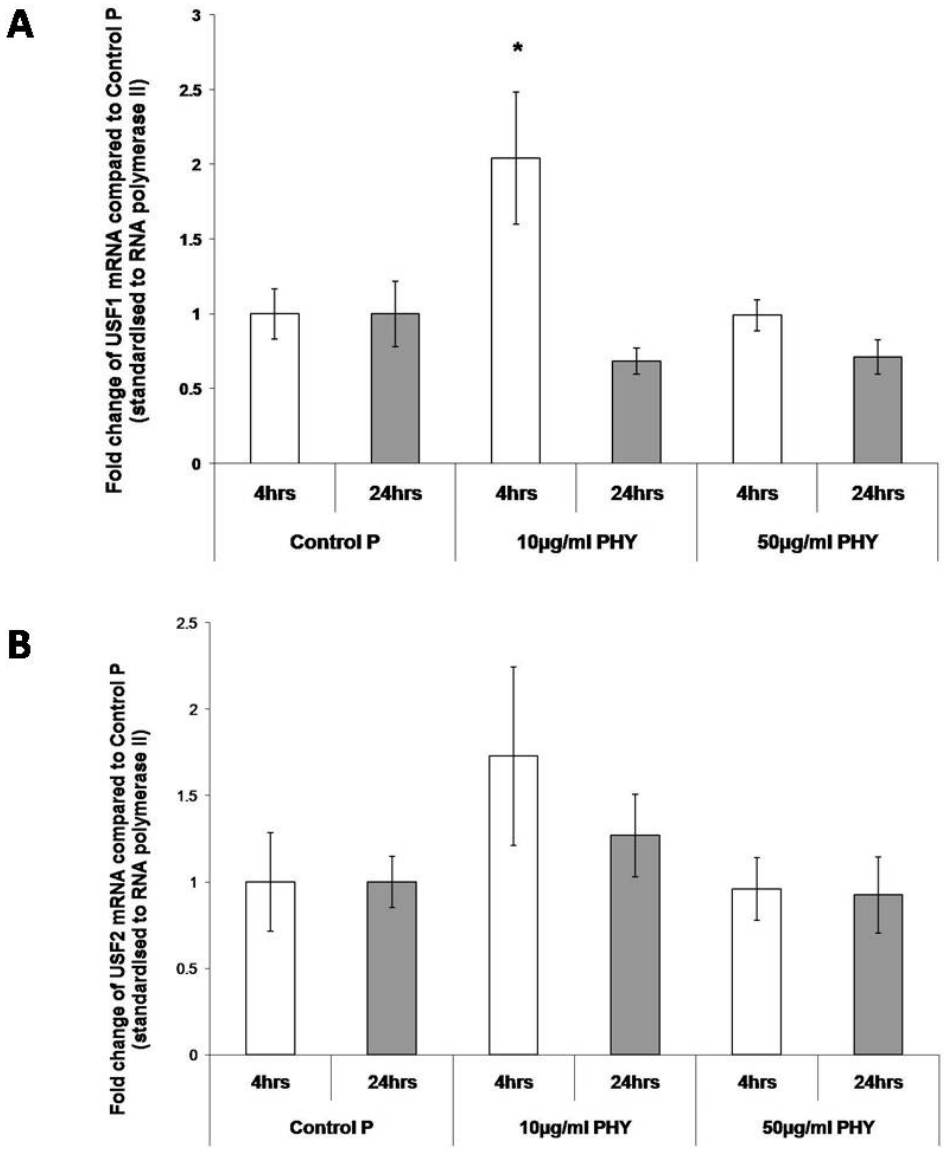

Figure 6.3 Modulation of the USF TFs by PHY. qPCR analysis of **(A)** USF1 and **(B)** USF2 mRNA expression in response to PHY treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 10µg/ml or 50µg/ml PHY, or vehicle control (Control P), for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of USF1 and USF2 were analysed using qPCR, standardized against the house keeping gene Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = *P = < 0.05*. (n=3).

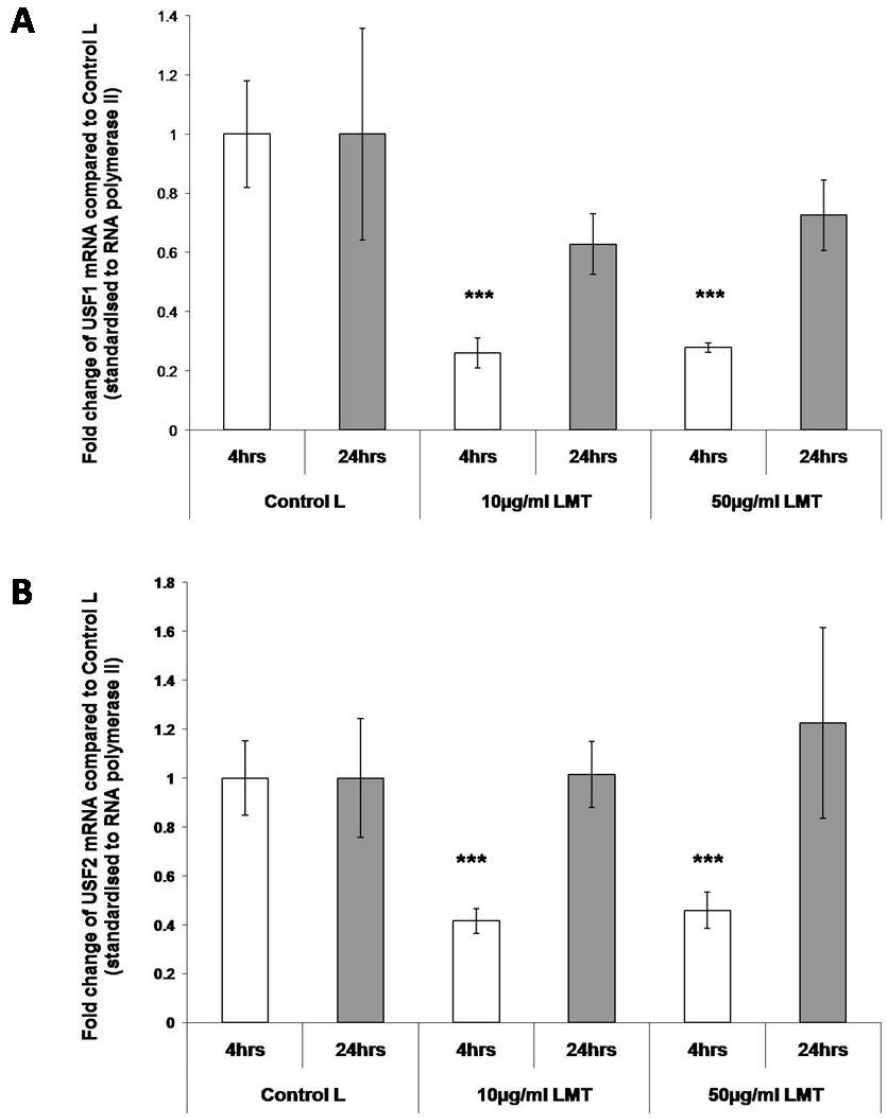

Figure 6.4 Modulation of the USF TFs by LMT. qPCR analysis of **(A)** USF1 and **(B)** USF2 mRNA expression in response to LMT treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 10µg/ml or 50µg/ml LMT, or vehicle control (Control L), for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of USF1 and USF2 were analysed using qPCR, standardized against the house keeping gene Pol II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: *** = $P = < 0.001$. (n=3).

6.4.3 Modulation of USF1 & USF2 location in human SH-SY5Y cells following ACD treatment.

6.4.3.1 Localisation of USF1 & USF2 in human SH-SY5Y cells.

In chapter 5, ACD treatment was found to have limited impact upon the localisation of the NRSF isoforms. Due to the postulated roles of USF proteins in seizure, it was important to extend this investigation, to test whether or not the ACDs had an impact on USF localisation. To detect USF1 and USF2, I opted to use antibodies previously employed by collaborators, Park & Russo as they used these antibodies in western blotting (both in untreated neurons and following siRNA knockdown of USF proteins), and in immunofluorescence (Park & Russo., 2008). USF1 and USF2 proteins are 43kDa and 44kDa in size, respectively, and in SH-SY5Y cells, the USF2 antibody detects a band at approximately 40kDa (Figure 6.5). In addition, the USF2 antibody detected two further bands, over 50kDa in size, however only the lower ~40kDa band was detectable in other cell line extracts tested within the lab (including JAr cells, HaCat cells and HeLa cells). This USF2 antibody has been shown to be specific by other groups, as this specific band is markedly reduced following USF2 knockdown (Park & Russo., 2008). The additional bands observed in the western blot may well be splice variants such as USF2b (Viollet *et al.,* 1996).

Intriguingly, the USF1 antibody failed to detect a band at the appropriate 43kDa size, but did detect a band at approximately 52kDa, which was also detected by the USF2 antibody. These ~50kDa bands have previously been detected in western blots with other USF1 and USF2 antibodies, by other groups (West *et al*., 2004) suggesting

that they are not, non-specific binding, but perhaps detecting splice variants of the two USF proteins. Furthermore, the USF1 antibody detected a band at approximately 95kDa, which was also detected by West *et al*., in human K562 nuclear extracts (West *et al*., 2004). Park & Russo, tested the specificity of the USF1 antibody employed here, via siRNA knockdown of USF1, noting a reduction in USF1 protein as detected by western blotting (Park & Russo., 2008).

Using confocal microscopy and immunofluorescence, the USF1 antibody was found to detect a nuclear protein in untreated SH-SY5Y cells (Figure 6.6), consistent with the published nuclear localisation of USF1 in cultured neurons (Park & Russo,. 2008). This nuclear staining of USF1 was found in all untreated cells.USF2 was found to be predominantly cytoplasmic, with some nuclear staining, in untreated SH-SY5Y cells (Figure 6.6). These findings are supportive of those found by Park & Russo, who found USF2 to exhibit a more cytoplasmic distribution in cultured rat trigeminal ganglia (Park & Russo., 2008). Due to the consistencies with previous findings, I continued to use the USF1 and USF2 antibodies, to determine the impact ACD treatment had, if any, on USF protein localisation.

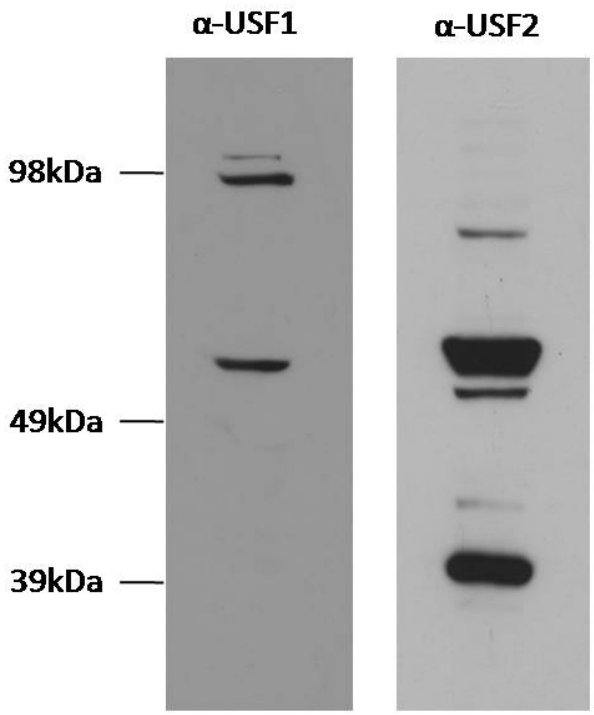

Figure 6.5 Western blotting of human SH-SY5Y cells extract with USF1 and USF2 antibodies. Human SH-SY5Y cell extract probed with the anti-USF1 antibody and anti-USF2. The USF2 antibody detects a band at the expected 44kDa, as well as a higher band above 51kDa. This higher band is also detected by the USF1 antibody.

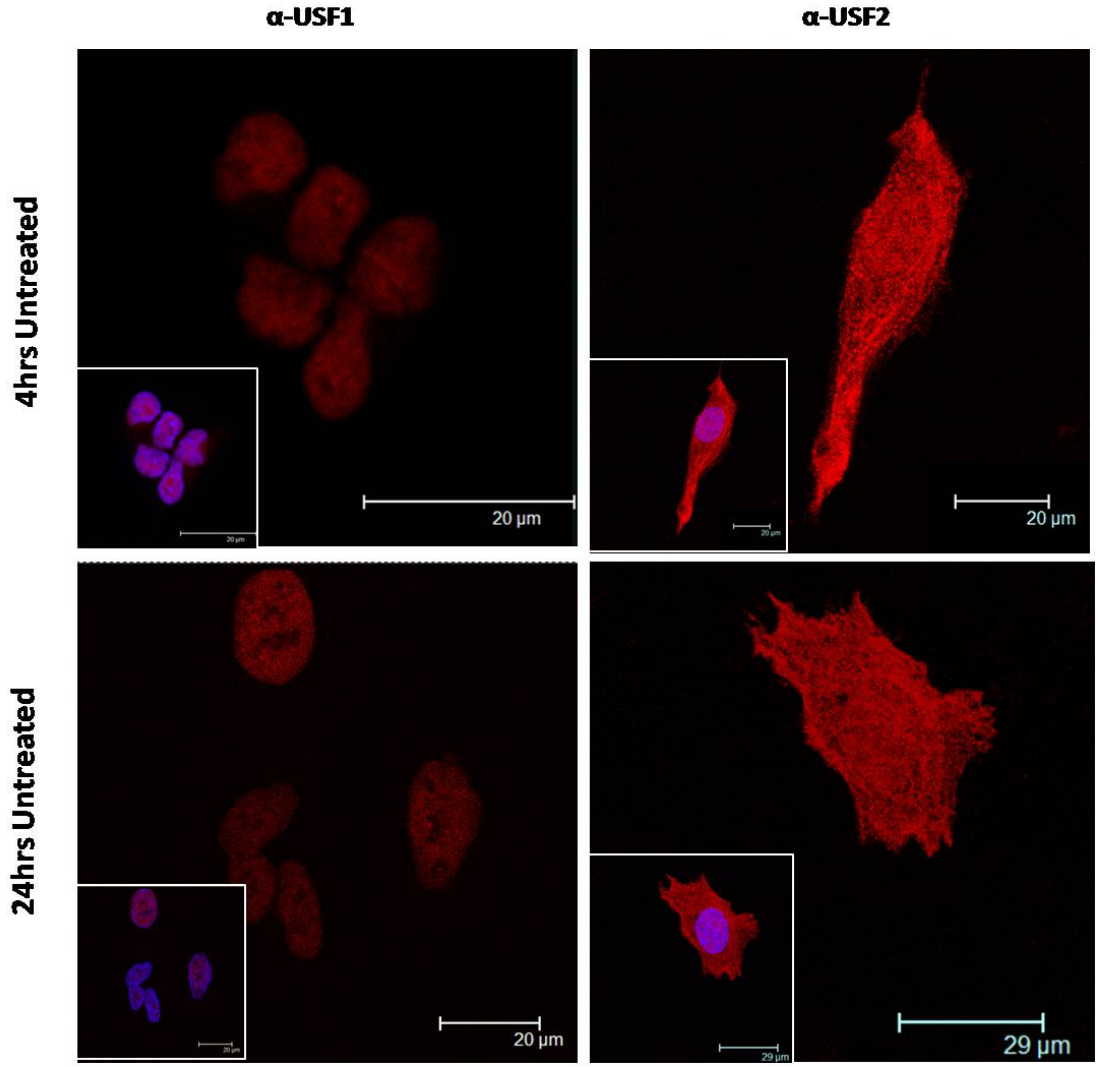

Figure 6.6 Immunofluorescence staining of USF1 and USF2 in human SH-SY5Y cells. Untreated human SH-SY5Y cells were stained with the anti-USF1 or anti-USF2 antibodies. Both primary antibodies were recognized with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. USF1 is shown to be nuclear, whilst USF2 exhibits predominantly cytoplasmic staining.

6.4.3.2 CBZ, PHY and LMT has limited impact upon USF1 and USF2 localisation in human SH-SY5Y cells.

As aforementioned, USF1 was found to be predominantly nuclear in untreated cells. This nuclear staining was found in all cells, including those exposed to the ACDs, and to the corresponding vehicle controls. ACD treatment had thus little impact upon USF1 localisation. Both 50µg/ml CBZ and the vehicle control (Control C), elicited exclusive nuclear staining of USF1 at both 4hrs and 24hrs treatment time-points (Figure 6.7), mirroring that observed in untreated cells (Figure 6.6). Similarly, USF1 staining was found to be fully nuclear following both 4hrs and 24hrs 50µg/ml PHY (Figure 6.8), which matched the distribution of both untreated cells (Figure 6.6) and vehicle control (Control P) treated cells (Figure 6.8). Finally, LMT treatment also had little impact upon USF1 localisation, with both vehicle control (Control L) and 50µg/ml LMT exhibiting fully nuclear USF1 localisation, at both 4hrs and 24hrs (Figure 6.9). Whilst USF1 distribution between nucleus and cytoplasm remained constant following LMT treatment, 24hrs 50µg/ml LMT did induce a modest change, with distinct nuclear aggregations observed (Figure 6.9).

The response of USF2 to both pro-convulsant and ACD treatment, in terms of mRNA expression, was found, in general, to mirror that of USF1. This consistency in the response of USF2 and USF1 to ACD treatment was also found to be the case with regards to modulation of USF2 localisation, with ACD treatment having little impact upon USF2 localisation in SH-SY5Y cells. USF2 was found to be distributed throughout the cell, with mostly cytoplasmic staining, and some nuclear (Figure 6.6). This predominantly cytoplasmic distribution of USF2 was also found in cells exposed to

either 4hrs vehicle control for CBZ treatment (Control C) or 4hrs 50µg/ml CBZ (Figure 6.10). In contrast, the more prolonged 24hrs treatment with both Control C and 50µg/ml CBZ resulted in a more intense nuclear USF2 staining pattern compared to untreated cells, with some cytoplasmic staining remaining (Figure 6.10). Whilst this data is not quantitative, the stronger nuclear staining observed may indicate that the vehicle control can induce a shift in USF2 localisation, whilst CBZ has no noticeable additional impact.

PHY and its vehicle control (Control P), evoked a similar response in USF2 localisation to CBZ and Control C. Cells exposed to either 4hrs Control P or 4hrs 50µg/ml PHY, exhibited predominantly cytoplasmic USF2 staining (Figure 6.11), matching that of untreated cells. In contrast, 24hrs exposure to Control P or 50µg/ml PHY induced a more nuclear USF2 distribution, with some cytoplasmic USF2 remaining (Figure 6.11). This indicates that prolonged exposure to the vehicle results in a shift in USF2 sub-cellular distribution, which appears unaffected by the ACD PHY.

Intriguingly, the vehicle control for LMT, Control L, has a higher concentration of DMSO, compared to Control P, and induced the same shift in USF2 localisation, to a predominantly nuclear distribution, within 4hrs exposure (Figure 6.12). This predominantly nuclear pattern (with some cytoplasmic staining) was also exhibited at 24hrs Control L, and following both 4hrs and 24hrs 50µg/ml LMT (Figure 6.12). Again, this reveals that the vehicle has a profound impact upon USF2 distribution, whilst the actual ACD, has no further effect.

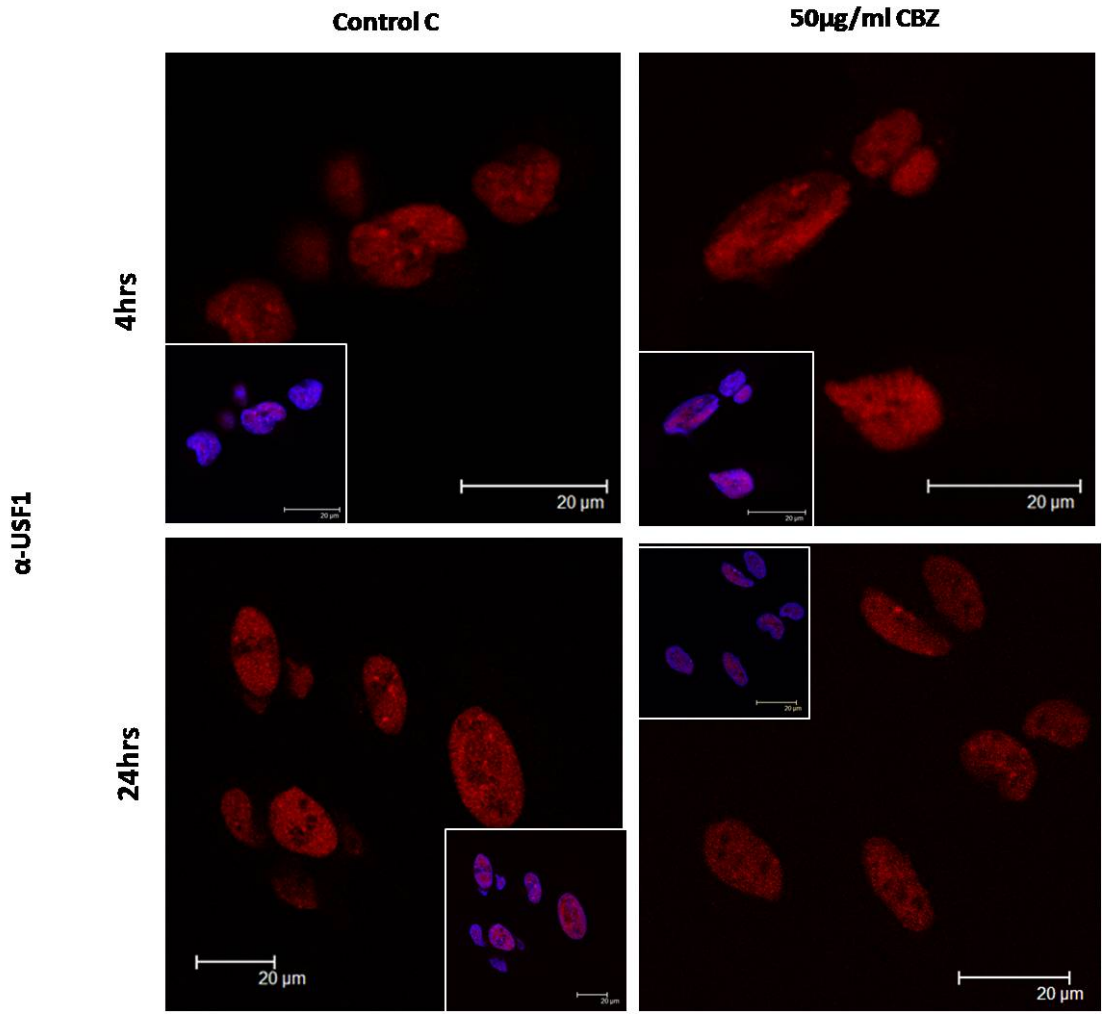

Figure 6.7 Immunofluorescence staining of USF1 in response to CBZ treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml CBZ or vehicle control (Control C). Cells were stained with the anti-USF1 antibody and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. USF1 is shown to be nuclear in Control C and CBZ treated cells. (n=2).

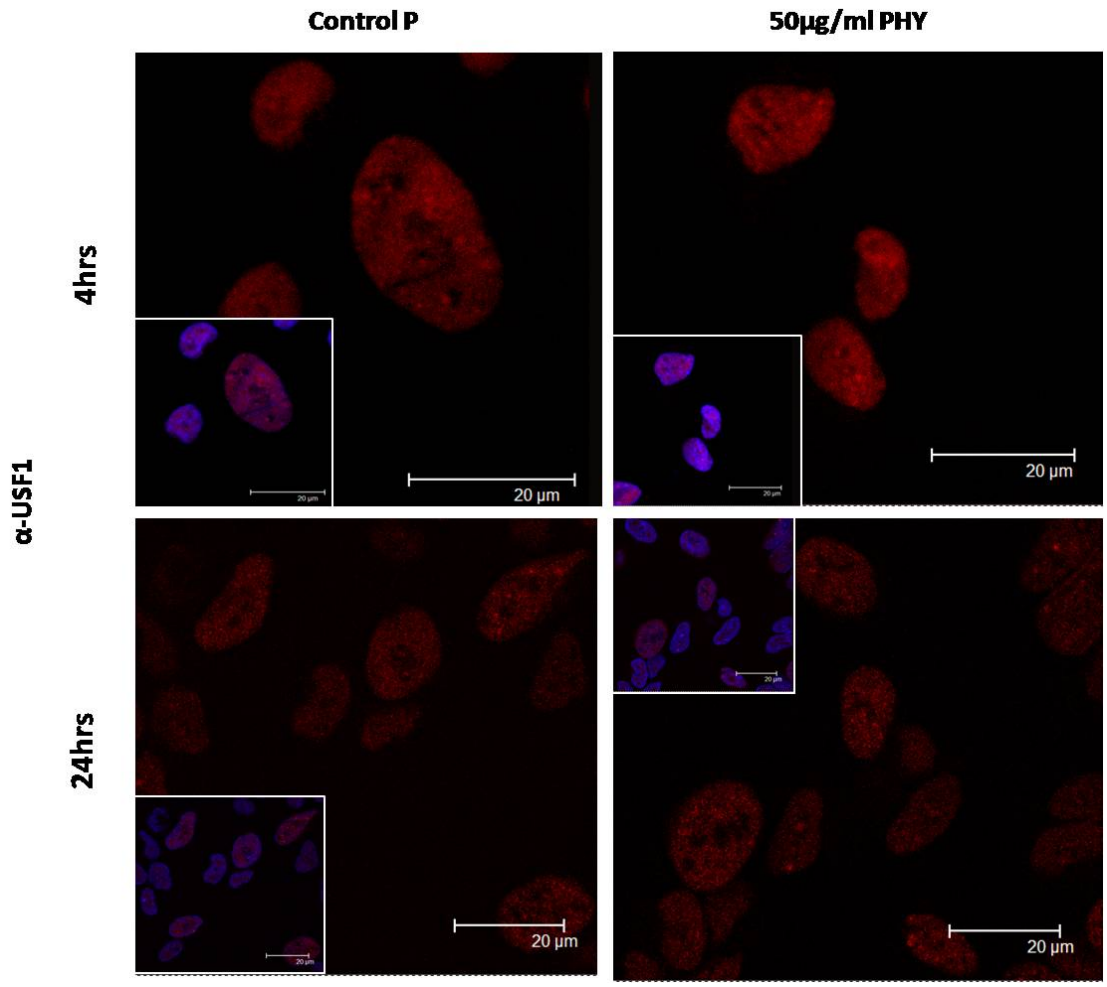

Figure 6.8 Immunofluorescence staining of USF1 in response to PHY treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml PHY or vehicle control (Control P). Cells were stained with the anti-USF1 antibody and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. USF1 is shown to be nuclear in Control P and PHY treated cells. (n=2).

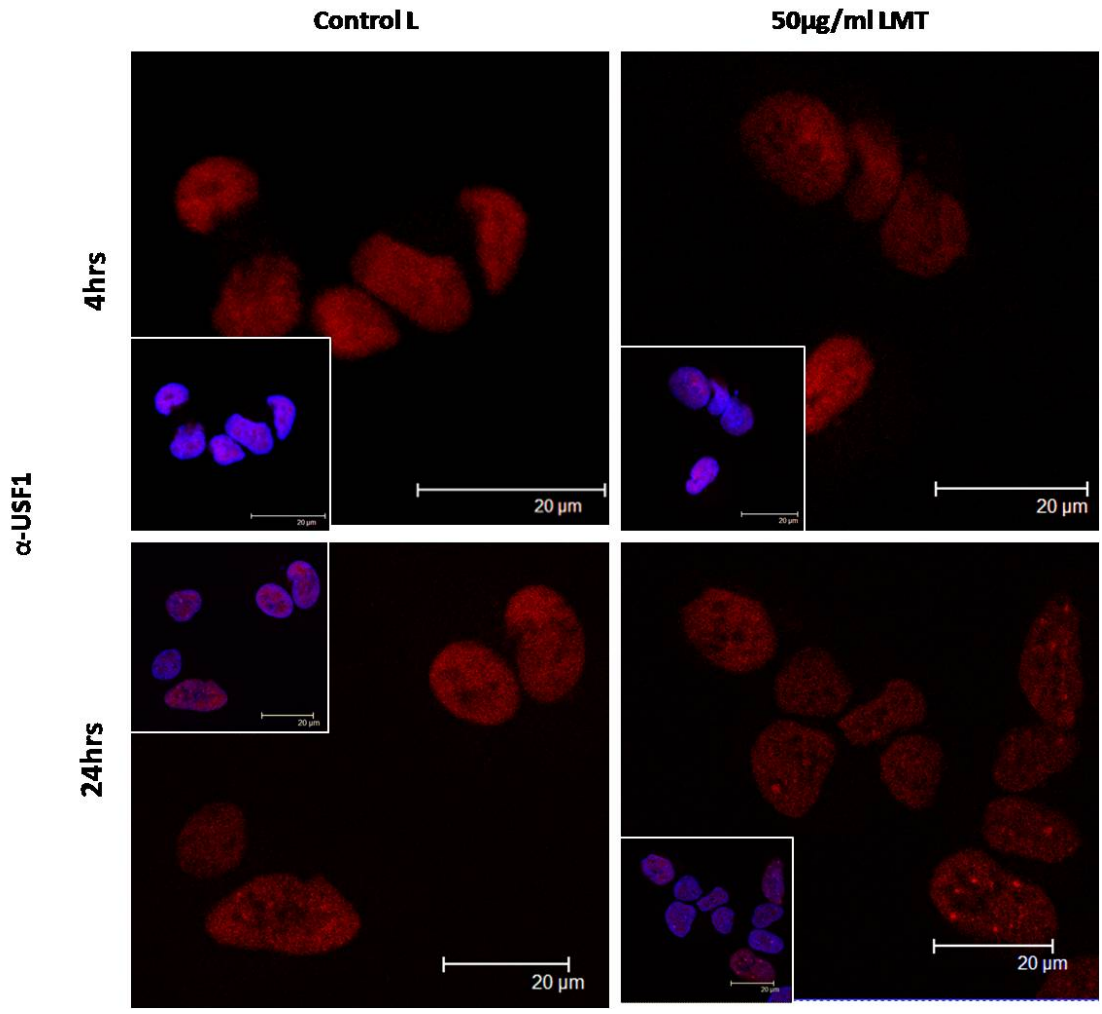

Figure 6.9 Immunofluorescence staining of USF1 in response to LMT treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml LMT or vehicle control (Control L). Cells were stained with the anti-USF1 antibody and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. USF1 is shown to be nuclear in Control L and LMT treated cells. (n=2).

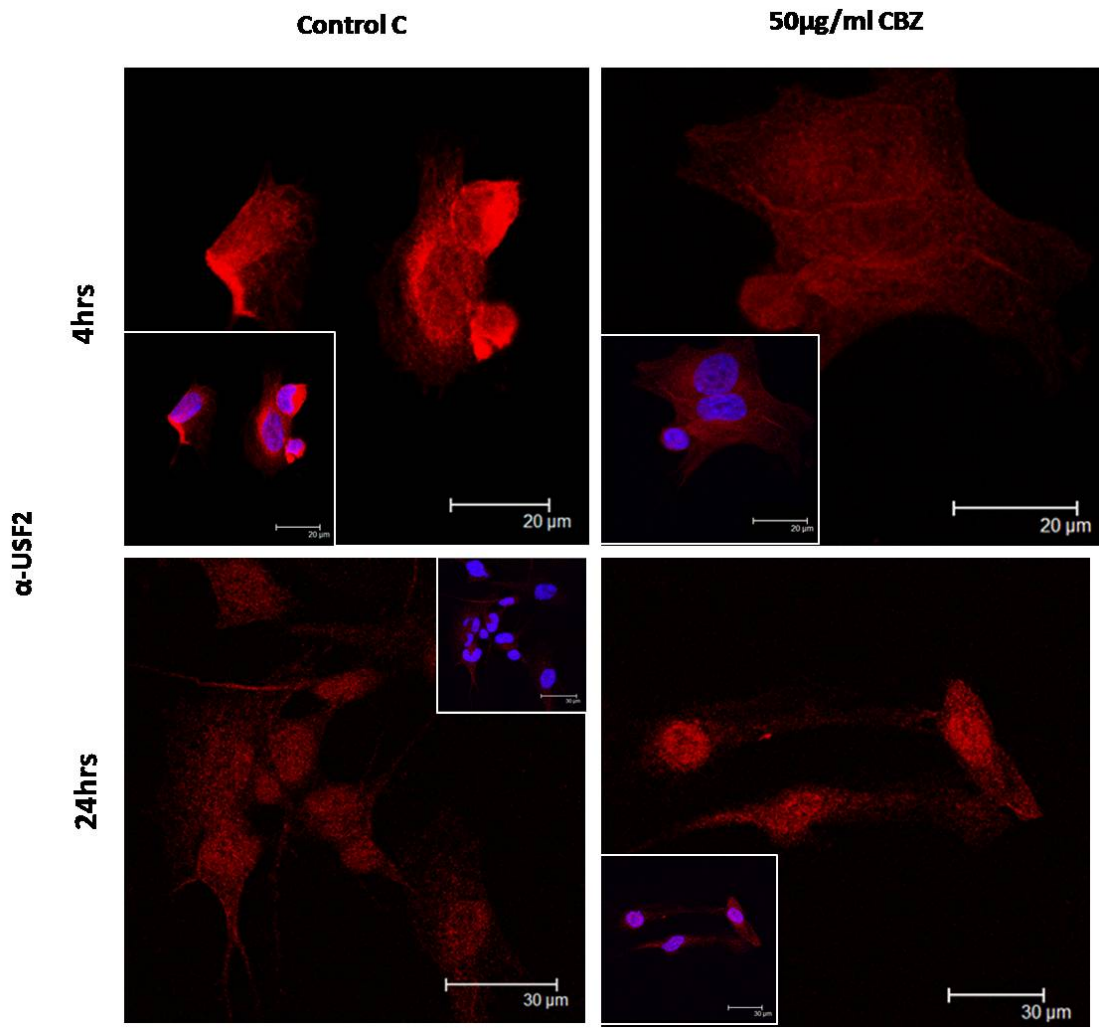

Figure 6.10 Immunofluorescence staining of USF2 in response to CBZ treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml CBZ or vehicle control (Control C). Cells were stained with the anti-USF2 antibody and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. CBZ treatment elicits similar staining patterns to vehicle control (Control C), with 4hrs treatment inducing predominantly cytoplasmic staining, and 24hrs giving more nuclear staining patterns. (n=2).

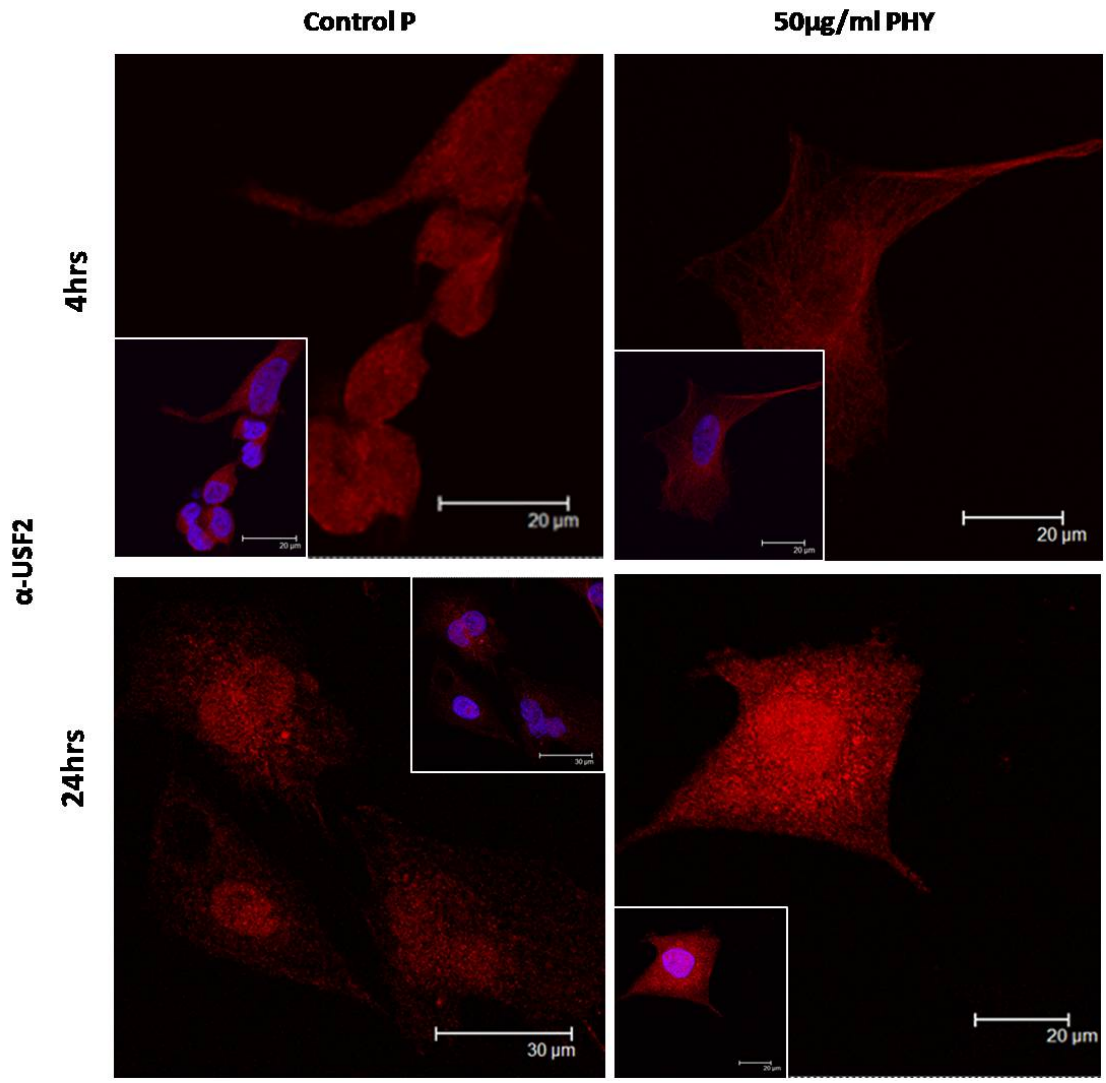

Figure 6.11 Immunofluorescence staining of USF2 in response to PHY treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml PHY or vehicle control (Control P). Cells were stained with the anti-USF2 antibody and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. PHY treatment elicits similar staining patterns to vehicle control (Control P), with 4hrs treatment inducing predominantly cytoplasmic staining, and 24hrs giving more nuclear USF2 staining patterns. (n=2).

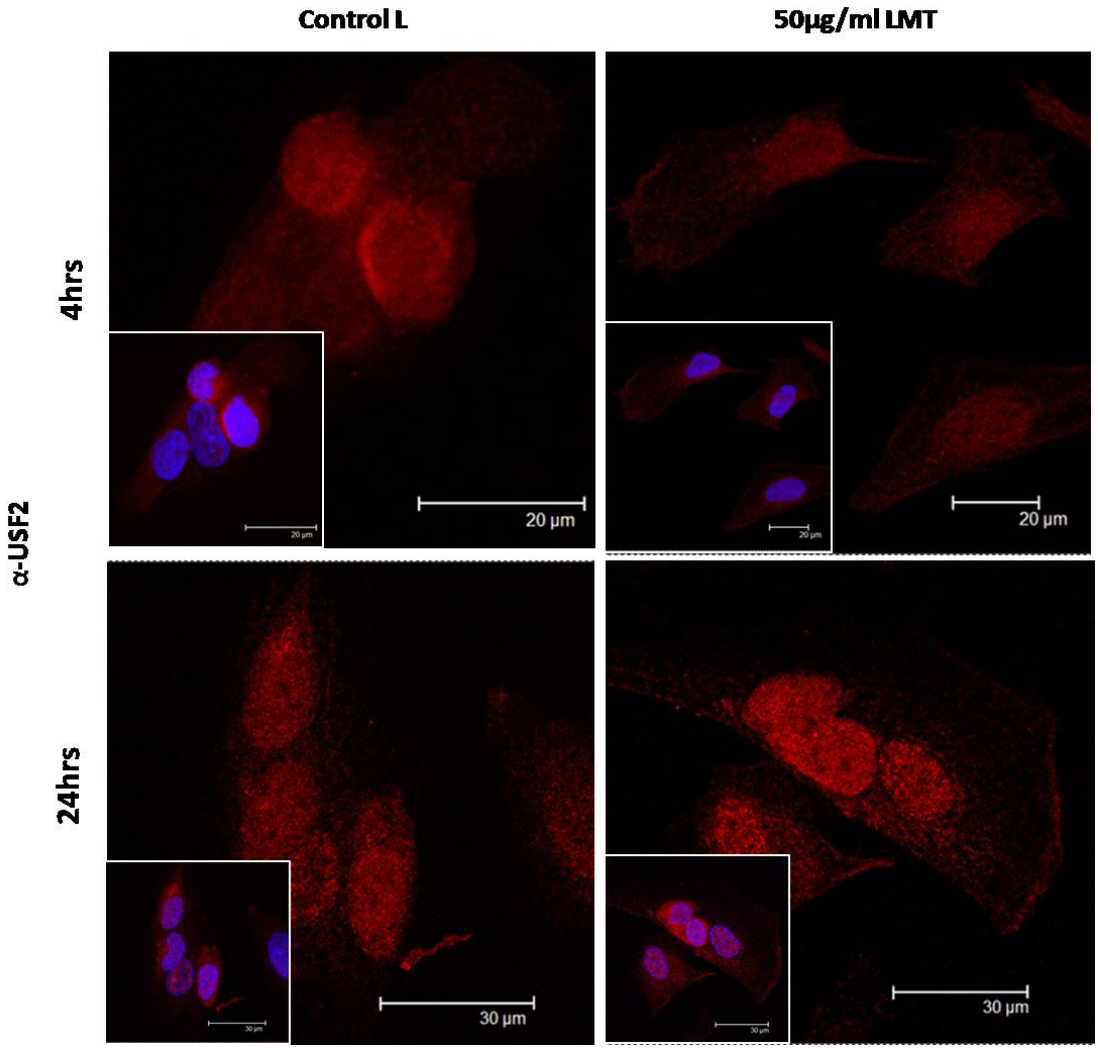

Figure 6.12 Immunofluorescence staining of USF2 in response to LMT treatment in human SH-SY5Y cells. Human SH-SY5Y cells were treated for 24hrs with either 50µg/ml LMT or vehicle control (Control L). Cells were stained with the anti-USF2 antibody and with Alexa 568 secondary antibody. Overlay images stained with DAPI and Alex 568, are given in the corners. LMT treatment induces similar staining patterns to vehicle control (Control L), with nuclear and cytoplasmic staining observed at both 4hrs and 24hrs time-points. (n=2).

6.4.3 Modulation of USF2 binding to TAC1 and NKB promoter regions in human SH-SY5Y cells by CBZ treatment.

ACD treatment has been shown to modulate USF1 and USF2 mRNA expression in human SH-SY5Y cells, but has little impact upon USF1 and USF2 localisation. This is analogous to the findings presented in Chapter 5, which revealed ACD treatment has a pronounced impact upon NRSF isoform mRNA expression, but limited impact upon NRSF isoform localisation. In addition, in chapter 5, ACD treatment was found to differentially modify NRSF binding to target regions, suggesting that these drugs can affect the function of NRSF in terms of recognising and binding to its DNA recognition motif, the NRSE. Here, I wished to explore whether or not a similar impact upon USF binding to its targets could be affected following ACD treatment. In previous chapters, USF2 has been shown to bind to the NKB and TAC1 promoter regions in the SH-SY5Y cells used here (chapters 3 and 4 respectively), and so I opted to explore USF2 binding. Unfortunately USF1 binding could not be explored in time for completion of this thesis, as experimental conditions could not be optimised in time. Furthermore, in chapter 3, the regulatory role of both USF proteins on the human NKB promoter was found to be abolished following CBZ. For these reasons I explored the impact of CBZ treatment upon USF2 binding to the human TAC1 and NKB promoter regions using a preliminary ChIP assay.

In a preliminary investigation, USF2 was found to bind to both the human TAC1 and NKB promoter regions in untreated SH-SY5Y cells, based upon a stronger PCR signal observed from the chromatin enriched following USF2 pull-down, compared to the non-specific background negative control; IgG (Figure 6.13). The vehicle control

(Control C) was found to elevate USF2 binding to the TAC1 promoter region, following 24hrs treatment, whilst USF2 binding to the NKB promoter region appears unchanged. 24hrs 50g/ml CBZ treatment was found to completely abolish USF2 binding to the TAC1 locus, with no USF2 binding observed (Figure 6.13). Interestingly, the data also suggests that USF2 binding to the NKB promoter is reduced following CBZ treatment, when compared to vehicle control treated and untreated cells. However it is important to note that the IgG negative control band was also elevated in CBZ treated cells, and so one cannot conclude CBZ is affecting USF2 binding to the NKB promoter. Despite this, this preliminary experiment has suggested that CBZ treatment can impair USF2 binding to the promoter regions of the pro-convulsant neuropeptide encoding gene TAC1, which may be consistent with its role as an anti-convulsant.

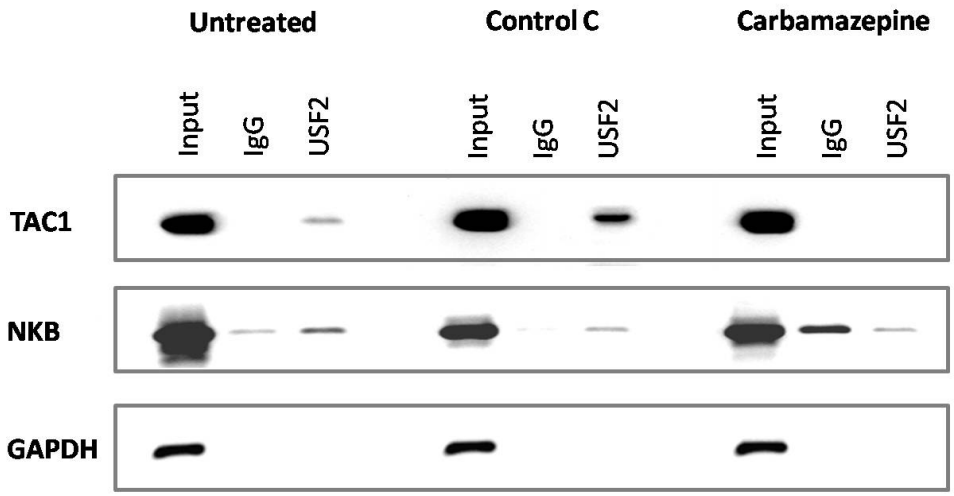

Figure 6.13. Modulation of USF2 binding to neuropeptide promoter regions following CBZ treatment in human SH-SY5Y cells. ChIP analysis of USF2 binding to human TAC1 or NKB promoter in SH-SY5Y cells treated with either 24hrs 50µg/ml CBZ, vehicle control (Control C) or untreated. IgG was included as a non-specific binding control (background). USF2 binding to the TAC1 promoter region was found to be diminished following CBZ treatment when compared to vehicle control. Binding is shown to be specific to the TAC1 of NKB promoter locus, as no binding is observed in the GAPDH PCR negative controls. (n=1).

6.5 Discussion

I have shown that the bHLH factors USF1 and USF2 regulate the pro-convulsant tachykinins NKB (chapter 3) and TAC1 (chapter 4), as have others (Paterson *et al*., 1995; Gerrard *et al*., 2005), in addition to other neuronal genes implicated in epilepsy such as BDNF (Tabuchi *et al.,* 2002b), $GABA_B$ receptors (Steiger *et al*., 2004) and KCC2 (Tornberg *et al*., 2005). Due to their regulation of such genes, I have postulated that the USF TFs are important in epilepsy, governing gene expression in response to seizure. In support of such a hypothesis, USF null-mice have been shown to be prone to spontaneous seizures (Sirito *et al.,* 1998), and a number of other bHLH factors are known to be modulated during seizure (Elliot *et al*., 2001).

As of yet, the response of USF1 and USF2 to seizure has yet to be clarified, but I have shown here, for the first time, that USF1 and USF2 expression is up-regulated following pro-convulsant challenge (KA) (Figure 6.1). This data suggests that the USF TFs are potentially modulated during seizure, and I propose that these TFs will respond to early changes brought about by seizure, in a similar manner shown by our lab, and others, of the NRSF isoforms (Palm *et al.,* 1998; Spencer *et al*., 2006). Consistent with such a model in which USF gene expression is induced during seizure, are the findings that the p38 mitogen-activated protein kinase signalling pathway is pivotal in seizure. USF1 is shown to be p38 activated (Galibert *et al*., 2001), and p38 immunoreactivity has been shown to be elevated in the rat hippocampus following 3hrs KA (Park *et al*., 2008). Furthermore, transgenic mice with a single copy disruption of the p38 gene (p38alpha(+/-), are found to have reduced seizure susceptibility and mortality rates (Namiki *et al*., 2007). Taken together, a potential role for USF in epilepsy becomes

more apparent, bringing forward the need to quantify USF expression in *in vivo* rodent seizure models in the future.

As USF1 and USF2 were found to be up-regulated during pro-convulsant (KA) challenge, similar to NRSF isoforms discussed in chapter 5, and the fact that the USF TFs regulate pro-convulsant neuropeptides, I sought to investigate the impact of ACD treatment upon USF expression, localisation and functional binding, akin to experiments undertaken in chapter 5. Interestingly the anti-convulsant treatment resulted in a number of parallels in the response of the USF TFs, compared to the NRSF isoforms, perhaps most notably being the lack of a response to ACD treatment in terms of TF localisation. Moreover, the response of USF mRNA expression to ACDs was found to be more consistent between CBZ and LMT treatment, compared to PHY, which mirrors that observed with the NRSF isoforms.

6.5.1 USF gene expression

As mentioned in chapter 5, the three ACDs chosen here all share a similar initial target, the sodium channel,(Kuo *et al*., 1998). Interestingly, each of these drugs have different structures, with each having other initial targets (e.g. LMT also targets high voltage gated calcium channels (Wang *et al.,*1996)) and each has differential downstream targets. These differences and similarities are reflected in the response of the USF genes to each of these drugs. CBZ was found to induce an exposure duration-dependent change in USF1 mRNA expression, with 4hrs treatment, but not 24hrs treatment, causing a highly significant reduction in USF1 mRNA (Figure 6.2a). USF2

also responded to CBZ with a marked reduction in USF2 mRNA expression observed at all concentrations and time-points tested (Figure 6.2b).

Intriguingly, LMT treatment induced a similar trend to CBZ in USF1 and USF2 modulation. As with CBZ treatment, LMT evoked an exposure-dependent modulation of USF1, with 4hrs, but not 24hrs LMT resulting in a significant reduction in USF1 expression. USF2 responded in a similar manner, with 4hrs LMT only inducing a reduction in USF2 expression (Figure 6.4b). Thus, following 4hrs treatment, both CBZ and LMT induce a significant reduction in both USF1 and USF2 mRNA expression, whilst also causing a significant up-regulation of full-length NRSF following (see chapter 5). As these two drugs have recently been shown to modulate BDNF in the rat cortex (Chang *et al*., 2009), a gene regulated by both USF and NRSF TFs, it is plausible that CBZ and LMT are modulating a common pathway.

One candidate pathway for modulation by CBZ and LMT is the p38 MAP kinase signalling pathway, which has been shown to be modulated by sodium channel blockade (a mechanism of action for both CBZ and LMT) via the drug Riluzole (Katoh-Semba *et al*., 2009). Riluzole was found to elevate p38 MAPK levels, which in turn elevated BDNF expression in rodent hippocampi (Katoh-Semba *et al*., 2009). Interestingly, blockade of N-type voltage gate calcium channels, which is one of LMT's mechanisms of action (Wang *et al*., 1996), reduces p38 production (Katoh-Semba *et al*., 2009). As stated earlier USF1 is activated by p38 (Galibert *et al.,* 2001) and so blocking p38 production, may in turn reduce USF1 expression, which would be consistent with our findings at 4hrs CBZ or LMT treatment. NRSF regulation may also be modulated by this pathway as p38 is found to be required for BDNF regulation (Katoh-Semba *et al*.,

2009), a well established NRSF regulated gene (Timmusk *et al*., 1999). It is plausible that NRSF is inhibited by this pathway, which would mean that reduced p38, via N-type Ca^{2+} channel blockade, would elevate NRSF expression, and potentially lead to suppression of BDNF production. Supportive of this are the recent findings the NRSF-mediated suppression of BDNF is activity dependent , changing in response to neuronal depolarisation (Hara *et al*., 2009). Consequently, an important future experiment would be to determine the role of the p38 MAPK signalling pathway on NRSF expression and function.

PHY treatment has been found to elicit quite different effects upon USF gene expression following the treatment conditions tested here. PHY treatment was found to have no significant effect on USF2 mRNA expression, but did result in an increase in USF1 expression following 4hrs 10µg/ml PHY (Figure 6.3). Furthermore, unlike CBZ and LMT, PHY treatment had no impact on NRSF expression (see chapter 5, Figure 5.3), but did repress sNRSF mRNA expression. This data implies that PHY may be modulating a separate signalling pathway to CBZ and LMT, inducing a different signalling cascade, and modulating different down-stream targets.

6.5.2 USF protein localisation

Despite invoking a differential change in USF1 and USF2 expression compared to CBZ and LMT, PHY did generate similar data to these drugs in terms of USF localisation in response to ACD treatment. All three drugs had little impact upon USF1 and USF2 localisation within the human neuroblastoma cells tested here, when compared to their corresponding vehicle controls. This is analogous to the lack of

response to these drugs from the NRSF isoforms. Interestingly, it was the vehicle controls which induced any observed change in USF localisation, with all three vehicle controls promoting a more nuclear localisation of USF2, compared to untreated cells. This USF2 nuclear localisation was also observed in cells exposed to the corresponding ACD treatment, indicating that the ACDs have no further impact upon USF2 localisation, compared to their vehicle controls. However, as stated in chapter 5, the immunoflourescence data is not quantitative, and so it would be of interest in future to measure USF2 protein levels in nuclear extracts in untreated and treated cells, to quantify the proposed changes in sub-cellular distribution. Furthermore, as proposed in chapter 5, it would be of interest to repeat the experiments with alternative vehicles for each ACD, to better derermine the impact of the ACDs on USF2 localisation.

As postulated in chapter 5, this lack of response of USF protein localisation, to ACD treatment (compared to vehicle controls) may be a result of these drugs maintaining a stable, normal neuronal phenotype, and in future, the more interesting experiment would be to stress the cells first with KA, monitor USF1 and USF2 localisation, and determine if the ACDs could restore any KA-induced change observed. In addition, it would be of interest to explore USF localisation in response to seizure, in an *in vivo* rat model, similar to that published by our lab for NRSF (Spencer *et al,* 2006). One note-worthy exception was found following 24hrs 50µg/ml LMT, with an increase in nuclear aggregations of USF1 found when compared to vehicle control (Control L) (Figure 6.9). These USF1 nuclear aggregations have previously been reported by other groups (Qyang *et al*., 1999) however their exact nature remains unclear. They may be promyelocytic leukemia (PML) nuclear bodies, which are known to accumulate TFs and

chromatin remodelling proteins such as CBP acetyltransferase and HDAC1 (Reviewed in Zimber *et al*., 2004). It would be important to determine what these nuclear aggregations are as this modulation is exclusive to LMT treatment, and as such may help determine the signal pathways LMT is targeting.

6.5.3 USF binding

The ability of a TF to govern gene regulation will not only rely on its localisation and expression, but also its ability to recognise and bind to its DNA recognition sequence. The USF TFs recognise and bind to a canonical E box sequence CANNTG. In previous chapters, USF binding to both the human NKB (chapter 3) and TAC1 (chapter 4) promoter regions was explored, with USF2, and not USF1, found to be bound to both promoter regions in SH-SY5Y cells. In contrast, in the SK-N-AS neuroblastoma cell line, USF1 was found bound to both promoter regions (Figure 3.7 and Figure 4.8). Due to the previously observed binding of USF2 only, to the TAC1 and NKB promoter regions, the effect of CBZ treatment on USF2 binding to these promoters was explored. In future, to determine the impact of such treatment on USF1 binding, one may opt to treat the SK-N-AS cell line, which exhibited USF1 binding to these promoter regions.

Previous reports have shown that the binding properties of the USF TFs, specifically USF1, to target E box motifs can be modulated by multiple signal transduction pathways and challenges, including the UV-stress induced p38 stress activate kinase pathway (Galibert *et al*., 2001), the phosphatidylinositol 3-kinase (PI3 kinase) pathway (Nowak *et al*., 2005) and the protein kinase A and C pathways (Xiao *et al*., 2002). Previous reports have shown that such pathways can be modulated by ACD

treatment. VPA treatment has recently been shown to activate the PI3 kinase pathway (Gurpur *et al.,* 2009), whilst CBZ has been shown to enhance the activity of the glutamate transporter 3 via the same pathway (Lee *et al.,* 2005). Due to these findings, I postulate that USF binding to target E box motifs could be modulated by ACD treatment. Indeed, in preliminary ChIP assays, CBZ treatment was found to reduce USF2 binding to the TAC1 promoter region, compared to vehicle controls and untreated cells (Figure 6.13), which supports the theory that ACDs can modulate USF2 binding to target regions. Such impairment of USF2 binding may be supportive of data presented in earlier chapters, whereby CBZ treatment was shown to significantly repress TAC1 mRNA expression (Figure 5.5), with the USF TFs shown to be an activator of TAC1 expression (Paterson *et al*., 1995). Thus CBZ not only reduces USF2 mRNA expression, but may also impair USF2 binding to the TAC1 promoter, leading, in part, to reduced TAC1 expression. This would be consistent with the role of an ACD to repress activation of a pro-convulsant neuropeptide, and to our knowledge, this is the first time this modulation has been reported. However, it should be stressed that these are only preliminary findings, that perhaps warrant further investigation in the future. Interestingly the decrease in USF2 binding to the TAC1 promoter region is in contrast to the elevated NRSF binding to the same regions, in the same CBZ treatment samples (Figure 5.15). This data, whilst preliminary, may indicate that a dynamic switch in TF recruitment to these pro-convulsant neuropeptide regions can occur under challenge, and may provide further support for the theory that NRSF isoforms and USF1 and USF2 may be competing for binding privileges at these promoter loci. It would therefore be of interest to explore USF binding to these loci, in response to NRSF variant over-

expression, and vice versa, to fully explore the relationship between USF and NRSF mediated regulation of these neuropeptide genes. Interestingly at the 24hrs time-point tested in ChIP assays, both USF2 and NRSF are found to be reduced following 50μg/ml CBZ treatment, and so it would be of interest to explore binding of these TFs following 4hrs CBZ, as whilst USF2 mRNA expression is reduced, NRSF expression is enhanced. This would enable us to explore this proposed competition for binding privileges, much like the over-expression study I alluded to.

To summarise, the data presented here suggests that USF1 and USF2 are modulated following pro-convulsant challenge in a human neuroblastoma cell line, and I postulate that these TFs are important factors bringing about dynamic changes in gene expression patterns, as an initial response to seizure. In addition, these findings indicate that ACD treatment targets and modulates the USF regulatory system. The drugs tested here modulated USF gene expression and possibly binding to target neuropeptide regulatory regions. These findings provide new evidence for the function of these ACDs in modulating distinct TF regulatory systems, which are implicated in seizure progression. The findings also support earlier discussions regarding an inherent relationship between the USF and NRSF regulatory systems, which appear to have an important role in the molecular mechanisms governing epilepsy.

CHAPTER 7: A distinct role for NRSF variants in modulation of epilepsy associated gene expression – revealed by microarray analysis

7.1 Introduction

Altered expression of genes such as BDNF or the pro-convulsant neuropeptide SP is associated with early changes in rodent models of status epilepticus and may be a marker of a generalised cellular derangement that leads to immediate and longer-term changes in gene expression associated with epilepsy. As mentioned in previous chapters, NRSF isoforms are up-regulated in seizure (Palm *et al*, 1998; Spencer *et al.,* 2006), and are known to regulate both BDNF (Timmusk *et al*., 1999; Tabuchi *et al*., 2002a) and the SP encoding gene TAC1 (Quinn *et al.,* 2002; Spencer *et al.,* 2006; Greco *et al*., 2007; Howard *et al*., 2008; Reddy *et al*., 2009). Furthermore, in chapter 3, NRSF variants were also found to regulate the expression of a second proconvulsant neuropeptide, NKB. Consistent with a model that NRSF isoforms are important in seizure, chapter 5 revealed that these isoforms were differentially modulated by ACD treatment, and recently a mutation in the gene encoding RILP, which governs NRSF localisation, has been correlated with epilepsy (Bassuk *et al*., 2008).

It is well known that NRSF can alter chromatin structure and disturb epigenetic markers on many genes (reviewed in Ooi & Wood., 2007), including those encoding ion channels (Tapia-Ramirez *et al.,* 1997; Kuwahara *et al.,* 2003; Cheong *et al.,* 2005) and neuropeptides (Quinn *et al.,* 2002; Coulson *et al.,* 1999., Coulson *et al.,* 2000). This ability to alter epigenetic signatures on genes implicated in epilepsy could lead to long-term changes in their gene expression profiles, which might change the threshold for

further seizures. The latter would be consistent with metabolic changes, induced by the glycolytic inhibitor 2-deoxy-D-glucose (2-DG), modulating seizure activity via, in part, modulation of NRSF function (Garriga-Canut *et al.,* 2006). In addition, many patients suffering with epilepsy also experience cognitive disturbances likely to be indicative of the underlying brain dysfunction. Consistent with the importance of NRSF on expression of genes such as BDNF, our lab has recently demonstrated that variants in both BDNF and NRSF are important determinants of cognitive decline in the elderly (Miyajima *et al.,* 2008).

Whilst the function of NRSF is well established, the function of the truncated isoform sNRSF remains unclear. The truncated isoform has been shown to antagonise the action of full-length NRSF, inhibiting NRSF binding to the cholinergic gene NRSE (Shimojo *et al.,* 1999) and inhibiting NRSF silencing of the BDNF promoter (Tabuchi *et al*., 2002). Furthermore, the truncated isoform has been shown to act as an activator rather than a repressor of some genes regulated by NRSF (Shimojo *et al.,* 1999; Tabuchi *et al.,* 2002; Spencer *et al*., 2006). The truncated isoform is therefore postulated to function differently to full-length NRSF. Indeed, in chapter 4, I eluded to functional differences between the two variants, based on the differential cooperation with the USF proteins in regulating the rat TAC1 promoter, and differential regulation of the human synapsin I promoter has also been reported (Magin *et al*., 2002). Our lab has previously suggested that the truncated NRSF isoform is perhaps more important than full-length NRSF, in modulating the pro-convulsant neuropeptide TAC1, due to the elevated response of the TAC1 promoter to the truncated isoform in rat hippocampi (Spencer *et al*., 2006) and in human SK-N-AS cells in cooperation with USF proteins (Chapter 4).

Due to the apparent importance of both NRSF isoforms in modulating gene expression following seizure, I sought to investigate the impact of over-expressing both full length and truncated NRSF variants on global gene expression profiles, through a Microarray analysis approach. Spencer *et al.,* showed that NRSF isoforms are up-regulated as an early response to seizure in rat hippocampi, and I was interested in whether this early response could induce medium to long-term changes in gene expression. Consequently, I have explored the impact of relatively long-term over-expression of NRSF variants (24hrs and 48hrs over-expression), on global gene expression patterns via an Affymetrix microarray. In addition, Spencer *et al*., show that whilst the full length NRSF is expressed endogenously in control rat hippocampi, the truncated isoform is non-detectable, but is vastly up-regulated following 3hrs KA (Spencer *et al.,* 2006). For my experiment, I wanted a cell line model which would be consistent with rat hippocampi in terms of NRSF expression, and opted to utilise the human SK-N-AS cell line, shown in chapter 3 to express NRSF endogenously, but not the truncated isoform (Figure 3.2).

To perform in-depth analysis of the Affymetrix microarray dataset, the GeneGo software suite (http://www.genego.com) has been employed. The GeneGo suite incorporates the MetaCore^{TM} database (GeneGo Inc.) of documented protein-protein (gene-gene) associations and interactions, as well as a set of graphical and statistical tools to allow one to build networks and pathways based on known relationships between genes. MetaCore^{TM} is a manually curated database, compiling published interactions or associations between genes or proteins. MetaCore^{TM} provided a platform to identify differences between REEX1 and HZ4 over-expression, on gene expression

profiles and pathways. Furthermore, the GeneGo software suite provided a tool to generate an epilepsy disease network map, based on a manual collation of genes implicated in epilepsy. The microarray dataset was screened against this epilepsy network map, to uncover the impact of NRSF variant over-expression, on the expression of numerous genes with known or postulated associations with epilepsy. Finally, RT-PCR was employed to validate changes in gene expression detected by the Microarray.

7.2 Aims

- To explore global changes in gene expression following over-expression of full-length NRSF via the REEX1 expression construct, and a truncated construct analogous to sNRSF (HZ4), in human SK-N-AS neuroblastoma cells.
- To use GeneGo bioinformatic software to analyse the Microarray generated data, screening for similarities and differences between the REEX1 and HZ4 over-expression.
- To use GeneGo bioinformatic software to analyse the Microarray generated data, screening for modulation of genes associated with epilepsy.
- Validate the dataset via RT-PCR.

7.3 Methods

7.3.1 Cell culture, transfections and RNA extraction

Human SK-N-AS cells were cultured in 6-well plates as outlined in methods section 2.2.2.1.1 and transfected as described in methods section 2.2.4.2, delivering either the full-length NRSF expression construct REEX1, or the truncated variant, HZ4. Following transfection, cells were harvested for RNA extraction as outlined in methods section 2.2.3.1, at 0hrs, 24hrs or 48hrs post transfection time points.

7.3.2 Affymetrix Microarray Analysis

In order to analyse global changes in gene expression patterns, Affymetrix Microarray technology was utilised. A total of 10µg of RNA was processed as outlined in methods section 2.2.3.6, hybridized to Human Genome U133 Plus 2.0 arrays from Affymetrix, Inc. (Santa Clara, CA), and scanned on an Affymetrix GeneChip® scanner 3000 at 2.5µm resolution. Image data was analysed using Affymetrix GCOS software, and imported into Microsoft Excel. Data was filtered as outlined in methods section 2.2.3.6, through pairwise comparison of either 24hrs or 48hrs time points, REEX1 and HZ4 transfected samples, against corresponding 0hrs controls.

7.3.3 Bioinformatics – GeneGo MetaCore^{TM} analysis

Filtered data generated from the Affymetrix microarray analysis, was parsed into the online GeneGo data mining software suite (http://www.genego.com). The microarray dataset was screened against the manually curated MetaCore database, to provide in-depth analysis of the data, enabling us to extract functionally significance

from the dataset. In addition, one employed the GeneGo MapEditor Version 2.6.0 (GeneGo Inc), to generate an 'epilepsy network map', incorporating genes implicated in epilepsy, discovered via manual screening of published findings.

7.3.4 mRNA expression analysis – Validation of Microarray findings

In order to validate the dataset retrieved from the Affymetrix microarray, the expression of a small number of genes showing marked modulation, was carried out. The expression of the potassium channels HCN2 and HCN3, and the sodium channel SCN9a following REEX1 and HZ4 over-expression, was investigated using PCR as described in methods section 2.2.3.4, using the PCR primers set out in Table 2.2.1. Thermal cycle conditions for all, were as follows: initial denaturation: 95^{o}C for 2mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds, annealing 62^{o}C for 30 seconds and elongation: 72^{o}C for 1min; and a final elongation step of 72^{O}C for 2mins.

7.4 Results

7.4.1 Over-expression of REEX1 and HZ4 modulate differential changes in gene expression profiles

The regulatory function of NRSF has been well documented since its discovery over a decade ago (Chong *et al*., 1995; Schoenherr & Anderson., 1995), but that of its truncated isoform, sNRSF, has so far yet to be fully determined. The truncated NRSF isoform has been postulated to function by antagonizing the action of full-length NRSF or indeed act as an activator rather than a repressor of genes regulated by NRSF (Shimojo *et al.,* 1999; Roopra *et al.,* 2000; Tabuchi *et al.,* 2002). In Chapter 4, I eluded to the fact that the truncated variant may function differently to the full-length isoform, due to the exclusive cooperation of the truncated variant with USF proteins to enhance the wild-type rat TAC1 promoter activity. Due to these potential functional differences between the two isoforms, I wished to explore global changes in gene-expression profiles, following over-expression of full-length NRSF via the REEX1 construct, and the over-expression of a truncated version, analogous to sNRSF: HZ4.

Human SK-N-AS neuroblastoma cells were transfected with either REEX1 or HZ4, and harvested at 0hrs, 24hrs and 48hrs post transfection, with 0hrs considered to be a baseline time point (control). Harvested RNA was processed and analysed using an Affymetrix Microarray. The data generated from the microarray was parsed into the online computational platform MetaCore^{TM} for in-depth analysis. MetaCore^{TM} analysis revealed that the over-expression of full length NRSF (REEX1) and the over-expression of the truncated variant (HZ4), led to differential changes in gene expression profiles at

both 24hrs and 48hrs time points, when compared to the 0hrs baseline time point. For both 24hrs and 48hrs time points, MetaCore^{TM} revealed that the number of genes modulated (either increase or decrease in expression) uniquely by either REEX1 or HZ4 over-expression, was greater than those which were modulated by both (Figure 7.1). At 24hrs, 534 genes were exclusively modulated by HZ4 and 307 genes were exclusively modulated by REEX1, whereas only 165 genes were modulated by both constructs (Figure 7.1a). Similarly at 48hrs, 566 genes were uniquely modulated by REEX1, 381 genes uniquely modulated by HZ4 and only 111 were found to be modulated by both (Figure 7.1b). ($P = < 0.01$) (n=3). These findings support a theory that the truncated variant regulates, either directly or indirectly, a distinctly different set of genes compared to full-length NRSF.

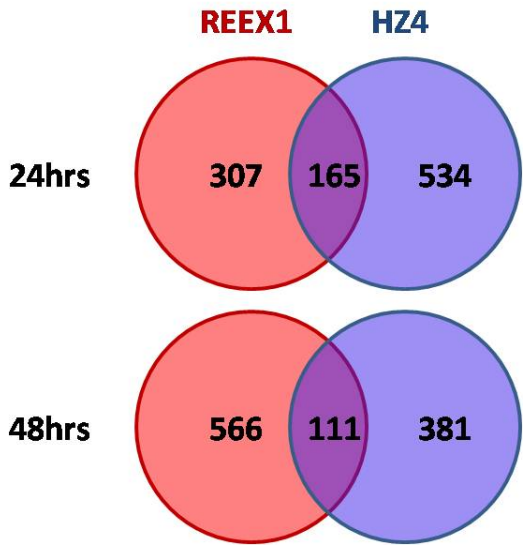

Figure 7.1. The number of genes modulated in response to the over expression of either REEX1 or HZ4. Venn diagram representing the number of genes found to be uniquely modulated following either 24hrs or 48hrs over expression of full length NRSF (REEX1) or the truncated variant (HZ4), in human SK-N-AS neuroblastoma cells ($P < 0.01$) (n=3). Genes found to be modulated by both NRSF (REEX1) or the truncated variant (HZ4) are represented by the overlapping sections.

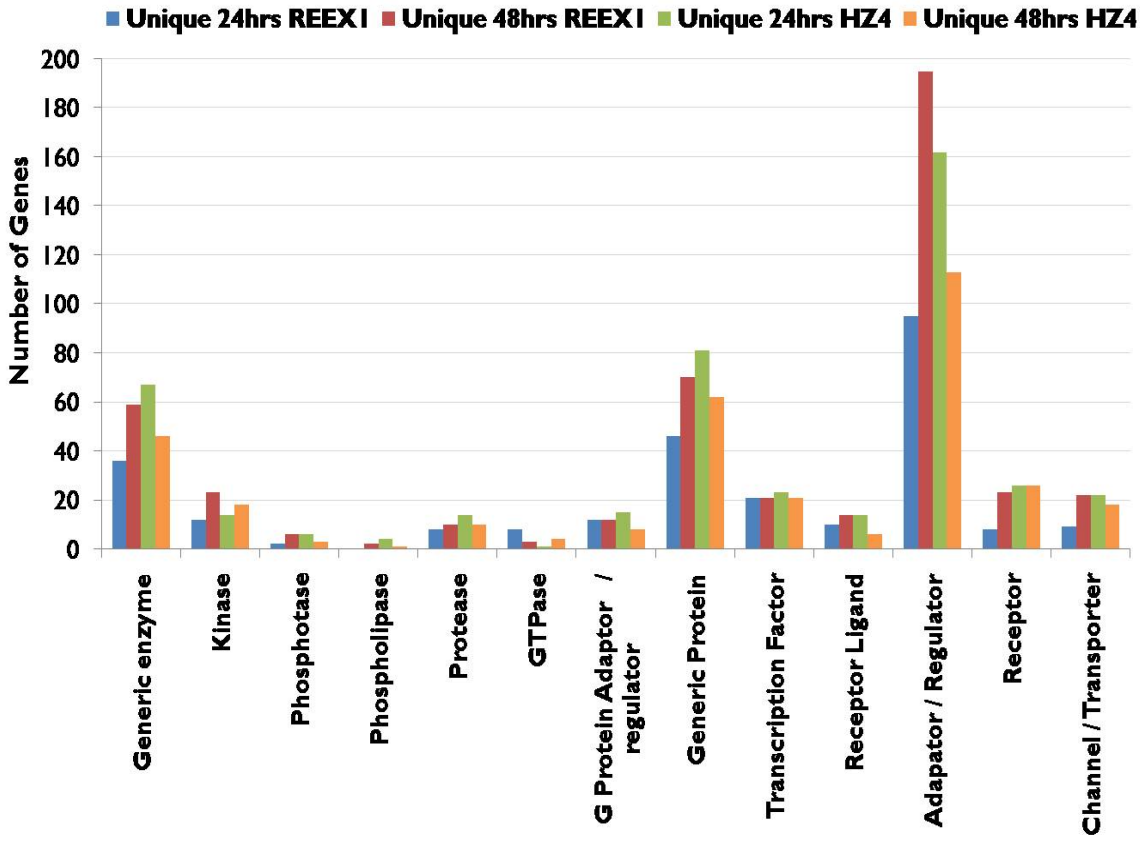

Figure 7.2. Over-expression of REEX1 and HZ4 modulate different types of genes. Bar chart represents the number of genes found to be uniquely and significantly modulated following over-expression of either 24hrs REEX1 (blue bar), 48hrs REEX1 (red bar), 24hrs HZ4 (green bar) or 48hrs HZ4 (purple bar), in human SK-N-AS neuroblastoma cells ($P < 0.01$) (n = 3). Gene descriptions or types where taken from MetaCoreTM.

MetaCore^{TM} provided the capability to probe the dataset to a greater extent, by subdividing the genes uniquely modulated by REEX1 or HZ4 over-expression (UNIQUE), into subgroups based on their function. These groupings included enzymes, adaptor proteins, transcription factors, receptors and channels, and transporters (Figure 7.2) ($P = < 0.01$) (n=3). A general trend was observed in 7 out of 13 groups and specifically in the three most gene-abundant groups (Adaptors/Regulators, Generic

Proteins and the Generic enzyme groups), with a marked increase in the number of uniquely modulated genes in each group following prolonged REEX1 over-expression (48hrs vs 24hrs) observed. In contrast, the more prolonged over-expression of HZ4 resulted in a decrease in the number of uniquely modulated genes (48hrs vs 24hrs), in the same 7 groups. This indicates therefore that the NRSF variants differentially modulate gene expression in a time dependent manner, and that duration of over-expression plays a role in determining gene expression pathways.

7.4.2 REEX1 and HZ4 over-expression modulates differential expression of genes implicated in epilepsy.

In chapters 3 and 4, the importance of the NRSF variants in the regulation of the pro-convulsant neuropeptides NKB and TAC1, respectively, was revealed and in chapter 5, these NRSF isoforms were found to be modulated by both KA and ACD treatment. The importance of these NRSF isoforms in epilepsy has been shown by both our group and others, and had been discussed in detail in previous chapters. To test our theory that NRSF isoforms are central to gene expression changes observed in epilepsy, I set out to explore and screen the microarray dataset for modulation of genes associated with epilepsy, following either REEX1 or HZ4 over-expression, as NRSF isoforms are known to be up-regulated during seizure (Palm *et al*., 1998; Spencer *et al*., 2006).

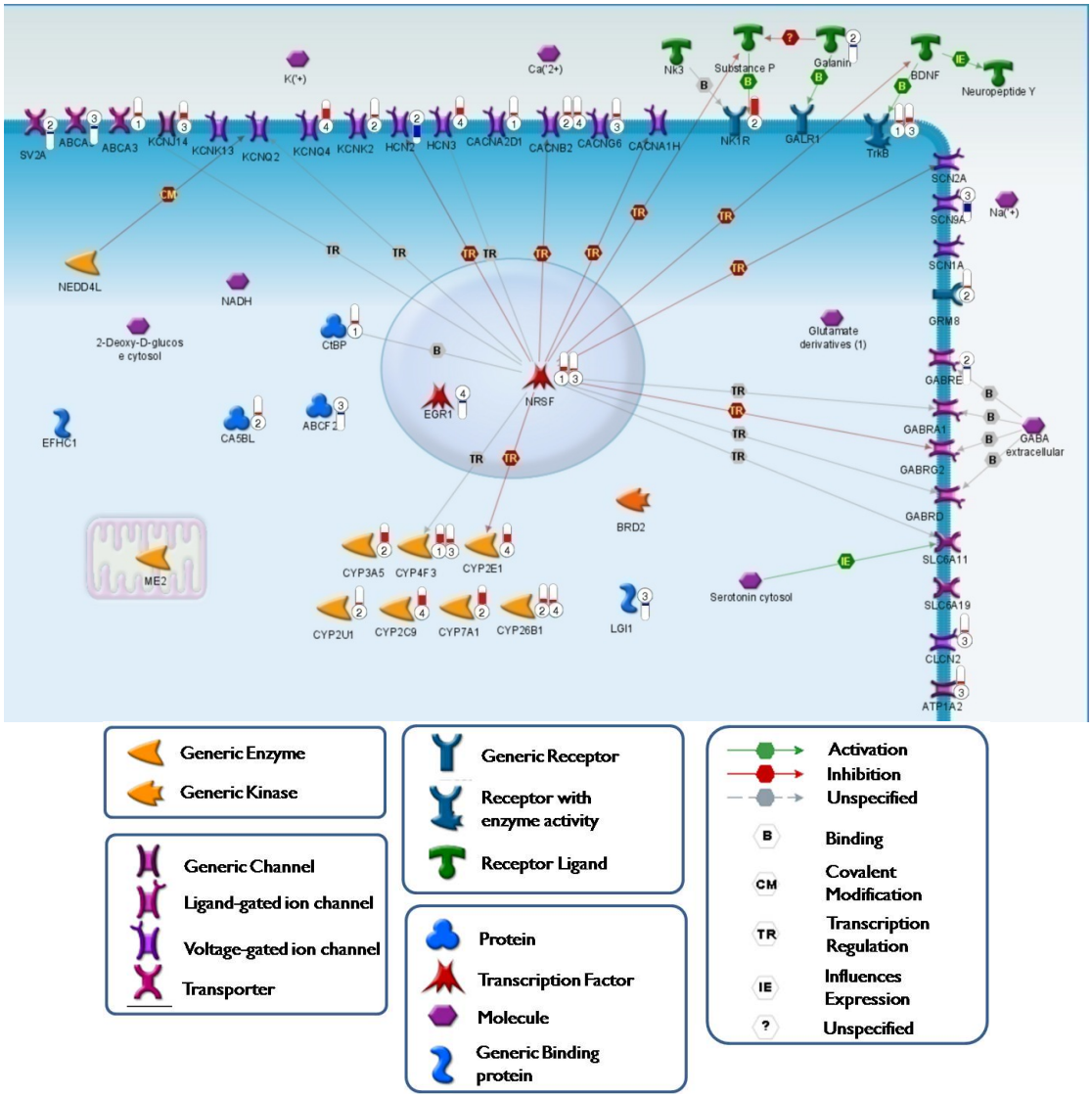

Figure 7.3. Modulation of genes associated with epilepsy by REEX1 and HZ4. Human SK-NAS neuroblastoma cells were transfected with full length NRSF (RE-EX1) or the truncated isoform sNRSF (HZ4) for 24 and 48hrs. Global changes in gene expression patterns were recorded via Microarray. The fold changes of genes associated with epilepsy, are plotted on the cellular diagram via the coloured rulers, with red indicating enhanced expression, and blue indicating suppressed expression. The different treatments are number: (1) 24hrs RE-EX1, (2) 48hrs RE-EX1, (3) 24hrs HZ4 and (4) 48hrs HZ4. All fold changed are significant at P = < 0.05.

I compiled a list of genes associated with epilepsy, through manually collating published associations, and supplementing this list with 'proposed' epilepsy associated genes, based on related genes having known associations with epilepsy. Using GeneGo's online software suite, I generated an 'epilepsy network map', which gave a diagrammatic representation of the 'epilepsy associated genes', and enabled MetaCore^{TM} to overlay the microarray data onto this map (Figure 7.3). This approach revealed that a great number of genes associated with epilepsy were found to be modulated following either REEX1 or HZ4 over-expression, or in some cases by both. This data is provided in Tables 7.1-7.4, and is represented on the epilepsy network map (Figure 7.3), by coloured rulers next to each gene symbol, with red colouration indicating up-regulation and blue colouration indicating down-regulation. Genes encoding ion channels were found to one of the most abundant type of 'epilepsy associated genes' modulated, including calcium channels such as CACNB2 (Table 7.1) ($P = < 0.001$), potassium channels such as HCN2 (Table 7.1) ($P = < 0.001$) and HCN3 (Table 7.3) ($P = < 0.05$), sodium channels such as SCN9a (Table 7.4) ($P = < 0.001$) and chloride channels such as CLCN2 (Table 7.3) ($P = < 0.05$) (all n=3).

Other noteworthy findings include the elevated expression of glutamate receptors GRM8/mGluR8 (Table 7.1) ($P = <0.01$) and GRIN2D (Table 7.3) ($P = 0.05$), following 48hrs REEX1 and 24hrs HZ4 over-expression, respectively (n=3) and the down-regulation of GABA receptors GABBR1 and GABRE following 24hrs and 48hrs REEX1 respectively (Table 7.2) ($P = < 0.05$) (n=3). Furthermore, a number of genes known to be important in anti-epileptic drug action, metabolism and resistance were modulated. This includes cytochrome p450 genes, such as CYP3A5 (Table 7.1) ($P = <$

0.001) (n=3), which modulates carbamazepine pharmacokinetics (Seo *et al*., 2006), and CYP2C9 (Table 7.3) (*P = < 0.01*) (n=3), known to metabolise the anti-convulsant drug phenytoin (Tate *et al.,* 2005; Mosher *et al.,* 2009). The GABA transporter SLC6A1, a target for the anti-convulsant drug tiagabine (Meldrum & Chapman., 1999), was up-regulated following both 24hrs REEX1 (Table 7.1) and 24hrs HZ4 (Table 7.3) (*P = < 0.05*) (n=3), and finally the Levetiracetam anticonvulsant target SV2a (Kaminski *et al.,* 2008; Lynch *et al.,* 2004), was found to be suppressed following 24hrs REEX1 over-expression (Table 7.2) (*P = < 0.01*) (n=3).

Gene	Description	Relevance to Epilepsy (Reference)	Fold Change	*P value*	Exposure
Calcium Channels					
CACNB2	Ca^{2+} channel, voltage-dependent, β2 subunit	Elevated in KA seizure models (Gastaldi *et al.,* 1998)	3.2	*0.0001*	48hrs
CACNA2D	Ca^{2+} channel, voltage-dependent, α2 / δ subunit 1	Target for ACDs Gabapentin (Gong *et al.,* 2001) and pregabalin (Joshi *et al*., 2006)	2.3	*0.044*	24hrs
Potassium Channels					
KCNT2	K^+ channel, subfamily T, member 2	*Proposed*	48.5	*0.0007*	24hrs
KCNE1/ISK	K^+ voltage-gated channel, Isk-related family, member 1	Mutation linked to QL syndrome (Splawski *et al*., 2000)	6.5	*0.004*	48hrs
KCNJ9/GIRK3	K^+ inwardly-rectifying channel, subfamily J, member 9	Ethosuximide inhibits GIRK channels (Kobayashi *et al.,* 2009)	4.0	*0.047*	24hrs
KCNQ1OT1	KCNQ1 overlapping transcript 1	*Proposed*	3.2	*0.002*	24hrs
KCNJ2/IRK1/Kir2.1	K^+ inwardly-rectifying channel, subfamily J, member 2	Kir2.1 expressing cells are elevated in hippocampus following KA (Kang *et al.,* 2008)	2.5	*0.03*	48hrs
KCNK2	K^+ channel, subfamily K, member 2	*Proposed*	2.5	*0.0009*	48hrs
GABA Transporters					
SLC6A1	Solute carrier family 6, member 1	Target for the ACD tiagabine (Meldrum & Chapman., 1999)	12.0	*0.029*	24hrs
GABA receptors					

GABRA4	GABA A receptor, alpha 4	GABRA4 promoter activity elevated in seizure (Roberts *et al*., 2005)	3.5	*0.045*	24hrs
Glutamate Receptors					
GRM8/mGluR8	Glutamate receptor, metabotropic 8	mGluR8 agonist has anticonvulsive effects (Jiang *et al*., 2007)	2.3	*0.0078*	48hrs
Carbonic Anhydrase					
CA9	Carbonic anhydrase IX	Carbonic anhydrase inhibitors act as anticonvulsant agent s *(Thiry et al.,* 2007*)*	2.0	*0.029*	24hrs
CA5BL	Carbonic anhydrase VB-like	Carbonic anhydrase inhibitors act as anticonvulsant agent s *(Thiry et al.,* 2007*)*	2.0	*0.033*	48hrs
Drug Metabolising Enzymes					
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	Modulates CBZ pharmacokinetics (Seo *et al*.,2006)	12.0	*0.001*	48hrs
Other					
NK1R / TACR1	Tackykinin-1 (TAC1) receptor	NK1 receptor antagonists reduces KA induced seizure activity (Zachrisson *et al*., 1998)	21.11	*0.0001*	48hrs
NRSF / REST	Neuron restrictive silencing factor /RE1-silencing transcription factor	Elevated in KA seizure models (Palm *et al*., 1998; Spencer *et al;* 2006)	3.72	*0.0001*	24hrs
NTRkB	BDNF/NT-3 growth factors receptor precursor	Elevated in SE models (Mudo *et al*., 1996;)	2.144	*0.014*	24hrs
CTBP2	C-terminal-binding protein 2	Implicated in molecular response of anti-epileptic Ketogenic diet (Garriga-Canut *et al.,* 2006)	2.0	*0.02*	24hrs

Table 7.1. Genes associated with epilepsy up-regulated following REEX1 over-expression. The over-expression of full-length NRSF, via the REEX1 expression construct, led to the significant increase in expression of genes listed, revealed by Affymetrix Microarray. Fold change, P values and over-expression duration are given. (n=3).

Gene	Description	Relevance to Epilepsy (Reference)	Fold Change	*P value*	Exposure
Calcium Channels					
CACNA1C	Ca^{2+} channel, voltage-dependent, L type, alpha 1C subunit	*Proposed*	12.1	*0.003*	48hrs
Potassium Channels					
HCN2	Hyperpolarization activated cyclic nucleotide-gated potassium channel 2	Reduced in KA seizure models (Powell *et al*., 2008)	14.9	*0.0004*	48hrs
KCNK13	K^+ channel, subfamily K, member 13	*Proposed*	13.0	*0.003*	24hrs
KCNB2	K^+ voltage-gated channel, Shab-related subfamily, member 2	*Proposed*	2.8	*0.005*	48hrs
KCNK17	K^+ channel, subfamily K, member 17	*Proposed*	2.0	*0.005*	48hrs
GABA Receptors					
GABBR1	GABA B receptor 1	*Proposed*	2.3	*0.035*	24hrs
GABRE	GABA A receptor, epsilon	The ACD Stiripentol acts directly on GABA A receptor, with reduced potentiation when epsilon subunits present (Fisher., 2009)	2.0	*0.047*	48hrs
Synaptic Vesicle Proteins					
SV2A	synaptic vesicle glycoprotein 2A	ACD Levetiracetam target (Kaminski *et al*., 2008; Lynch *et al.,* 2004)	2.1	*0.008*	24hrs
Other					
GAL	Galanin	Anticonvulsant neuropeptide (Lerner *et al.,* 2008)	2.3	*0.0005*	48hrs

Table 7.2. Genes associated with epilepsy down-regulated following REEX1 over-expression. The over-expression of full-length NRSF, via the REEX1 expression construct, led to the significant decrease in expression of genes listed, revealed by Affymetrix Microarray. Fold change, P values and over-expression duration are given. (n=3).

Gene	Description	Relevance to Epilepsy (Reference)	Fold Change	*P value*	Exposure
Calcium Channels					
CACNA2D	Ca^{2+} channel, voltage-dependent, α2 / δ subunit 1	Target for ACDs Gabapentin (Gong *et al*., 2001) and pregabalin (Joshi *et al*., 2006)	16.0	0.00002	24hrs
CACNG6	Ca^{2+} channel, voltage-dependent, gamma subunit 6	*Proposed*	2.8	0.001	24hrs
CACNB2	Ca^{2+} channel, voltage-dependent, beta 2 subunit	Elevated in KA seizure models (Gastaldi *et al.,* 1998)	2.0	0.0001	48hrs
Potassium Channels					
KCNJ9/GIRK3	K^+ inwardly-rectifying channel, subfamily J, member 9	Candidate gene for seizure susceptibility (Lohoff *et al.,*2005)	21.0	0.0003	24hrs
KCNQ4	K^+ voltage-gated channel, KQT-like subfamily, member 4	Activated by ACD retigabine (Schroder *et al.,* 2001)	13.0	0.001	48hrs
HCN3	Hyperpolarization activated cyclic nucleotide-gated K^+ channel 3	*Proposed: HCN gene family implicated in epilepsy (*Mistrik *et al., 2005)*	9.8	0.021	48hrs
KCNJ14/IRK4	K^+ inwardly-rectifying channel, subfamily J, member 14	*Proposed*	8.5	0.036	24hrs
KCNT2	K^+ channel, subfamily T, member 2	*Proposed*	4.9	0.007	24hrs
KCNE1	K^+ voltage-gated channel, Isk-related family, member 1	Mutation linked to QL syndrome (Splawski *et al*., 2000)	4.3	0.004	48hrs
KCNMB4	K^+ large conductance calcium-activated channel, subfamily M, beta member 4	variations infer susceptibility to sporadic epilepsy syndrome (Cavalleri *et al.,* 2007)	3.0	0.004	48hrs
Chloride Channels					
CLCN2	Chloride Channel 2	Susceptibility gene for childhood absence epilepsy (Everett *et al*., 2007) & idiopathic generalised seizure (Niemeyer *et al*., 2004)	2.0	0.0384	24hrs
GABA Transporters					
SLC6A1	Solute carrier family 6, member 1	Target for the ACD tiagabine (Meldrum & Chapman., 1999)	13.0	0.033	24hrs
GABA receptors					
GABRA5	GABA A receptor, alpha 5	Implicated in absence epilepsy (Lu *et al*., 2004)	2.6	0.022	24hrs
Glutamate Receptors					

GRIN2D	Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	*Proposed*	2.0	0.005	24hrs
Carbonic Anhydrase					
CA5	Carbonic anhydrase VA	Carbonic anhydrase inhibitors act as anticonvulsant agent s *(Thiry et al.,* 2007*)*	42.0	0.008	24hrs
Drug Metabolising Enzymes					
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	Metabolises the ACD phenytoin (Tate *et al*., 2005; Mosher *et al*., 2009)	13.0	*0.002*	48hrs
Other					
ATP1A2	ATPase, Na^+/K^+ transporting, alpha 2 polypeptide	ATP1A2 mutations noted in epilepsy (Gutierrez-Delicado *et al*., 2004; Gallanti *et al*., 2008)	3.482	*0.003*	24hrs
NRSF/REST	Neuron restrictive silencing factor /RE1-silencing transcription factor	Elevated in KA seizure models (Palm *et al*., 1998; Spencer *et al;* 2006)	2.144	*0.001*	24hrs
NTRkB	BDNF/NT-3 growth factors receptor precursor	Elevated in SE models (Mudo *et al*., 1996)	5.657	*0.041*	24hrs

Table 7.3. Genes associated with epilepsy up-regulated following HZ4 over-expression. The over-expression of the truncated variant, analogous to sNRSF, via the HZ4 expression construct, led to the significant increase in expression of genes listed, as revealed by Affymetrix Microarray. Fold change, P values and over-expression duration are given. (n=3).

Gene	Description	Relevance to Epilepsy (Reference)	Fold Change	*P value*	Exposure
Sodium Channels					
SCN9A	Na^+ channel, voltage-gated, type IX, alpha subunit	*Proposed*	10.6	*0.0003*	24hrs
Potassium Channels					
KCNK12	K^+ channel, subfamily K, member 12	*Proposed*	20	*0.008*	48hrs
KCNB2	K^+ voltage-gated channel, Shab-related subfamily, member 2	*Proposed*	3	*0.009*	48hrs
KCNC3/Kv 3.3	K^+ voltage-gated channel, Shaw-related subfamily, member 3	KV3 channel suppression causes seizures (Rudy *et al.,* 1999)	2.3	*0.016*	48hrs
KCNC4/Kv 3.4	K^+ voltage-gated channel, Shaw-related subfamily, member 4	Kv3.4 modulated by 4-aminopyridine in SE rats (Zahn *et al.,* 2008).	2	*0.0078*	48hrs
Other					
LGI1	leucine-rich, glioma inactivated 1	LGI1 mutations associated with epilepsy (Berkovic *et al.,* 2004)	3.2	*0.009*	24hrs
EGR1	Early growth response protein 1	Differentially expressed in absence seizure (Helbig *et al*., 2008)	2.0	*0.0001*	48hrs

Table 7.4. Genes associated with epilepsy down-regulated following HZ4 over-expression. The over-expression of the truncated variant, analogous to sNRSF, via the HZ4 expression construct, led to the significant decrease in expression of genes listed, as revealed by Affymetrix Microarray. Fold change, P values and over-expression duration are given. (n=3).

7.4.2 RT-PCR validation of Microarray data – HCN2, HCN3 & SCN9a

The microarray data indicated that over-expression of the NRSF variants led to the modulation of numerous genes. It was important however to validate changes in gene expression following NRSF variant over-expression, revealed by the microarray dataset. To this end, the modulation of expression of three ion channels HCN2, HCN3 and SCN9a was analysed via RT-PCR. The potassium channels HCN2 and HCN3 were analysed as they have both been previously identified as NRSF targets (Kuwahara *et al*.,

2003; Sun *et al*., 2005; Johnson *et al*., 2007), and thus by validating changes in expression of these two genes, and comparing our data against previous publications, greater strength and confidence could be given to the microarray dataset. In addition, a gene with no published regulatory association with NRSF - SCN9a, was also screened. Two related genes, SCN2a (Tapia-Ramirez *et al.,* 1997) and SCN8a (Drews *et al.,* 2007) have previously been shown to be NRSF regulated, and thus SCN9a may also be regulated by NRSF variants.

RT-PCR revealed a significant reduction in expression of the SCN9a gene in human SK-N-AS neuroblastoma cells, following 24hrs HZ4 over-expression compared to 0hr baseline time-points (Figure 7.4) ($P = < 0.001$) (n=3), when standardised against the house keeping gene RNA polymerase II (Pol-2). This is consistent with the change in SCN9a detected by the microarray. Similarly, the expression of the HCN2 gene was found to be significantly reduced following 48hrs over-expression of full length NRSF (REEX1), normalised against Pol-2 (Figure 7.5) ($P = < 0.05$) (n=3). This is also consistent with the microarray dataset, and supports previously published reports that HCN2 is repressed by NRSF (Kuwahara *et al*., 2003), based on the presence of a putative NRSE (Johnson *et al*., 2007). Finally the microarray dataset indicated an increase in HCN3 expression following 48hrs HZ4 over-expression. This was validated by RT-PCR, with 48hrs HZ4 over-expression leading to an increase in HCN3 expression, when compared to 0hrs baseline time-points, and standardised to Pol-2 (Figure 7.6) ($P = < 0.05$) (n=3).

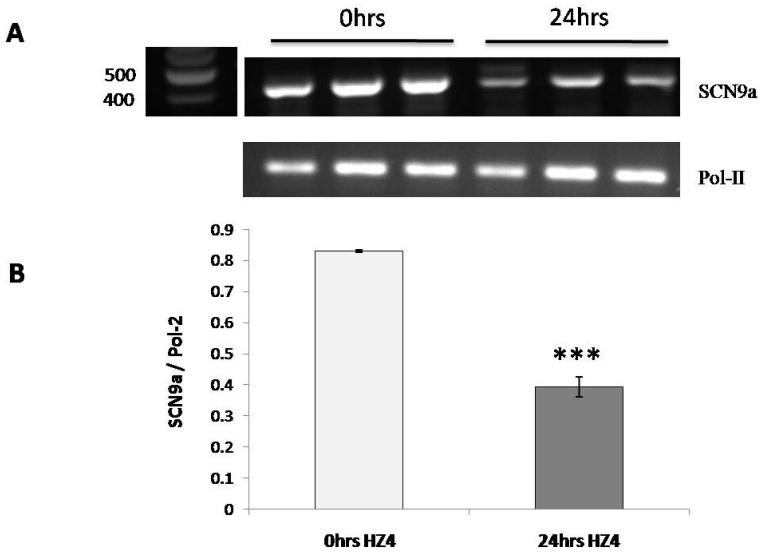

Figure 7.4. Over-expression of HZ4 represses SCN9a. 24hrs over-expression of the truncated NRSF construct (HZ4), led to the significant reduction of SCN9a mRNA levels. (A) RT-PCR of SCN9a and Pol-II following 0hrs and 24hrs HZ4 and (B) corresponding band intensities giving ratio of SCN9a to Pol-II. Human SK-NAS neuroblastoma cells were transfected with the HZ4 expression construct and were cultured for 0hrs or 24hrs before RNA extraction occurred. *** = $P < 0.001$. (n=3).

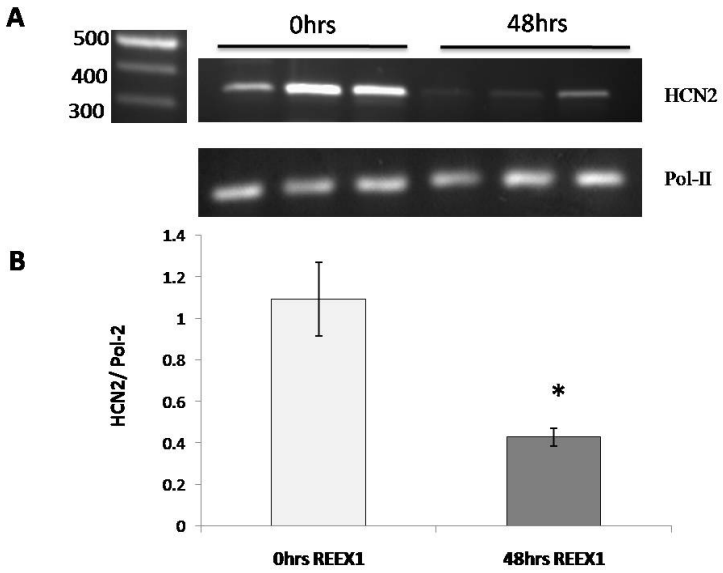

Figure 7.5. Over-expression of REEX1 represses HCN2. 48hrs over-expression of NRSF (REEX1), led to a significant reduction of HCN2 mRNA levels. Human SK-NAS neuroblastoma cells were transfected with the REEX1 expression construct and were cultured for 0hrs or 48hrs before RNA extraction occurred. (A) RT-PCR was used to detect endogenous levels of HCN2 and Pol-II, and (B) corresponding band intensities comparing HCN2 to Pol-II are given. * = P < 0.05. (n=3).

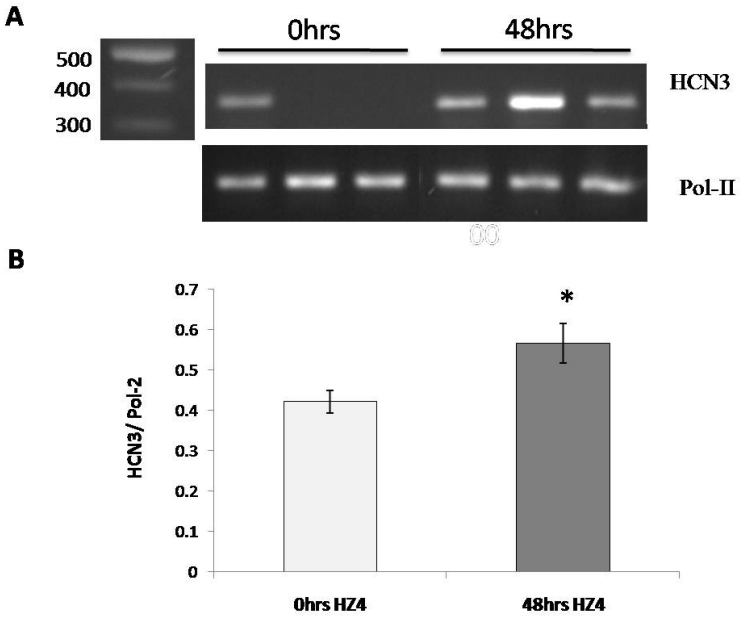

Figure 7.6. Over-expression of HZ*4* elevates HCN3. 48hrs over-expression of the truncated variant construct (HZ4), led to the significant increase in HCN3 mRNA levels. (A) RT-PCR of HCN3 and Pol-II following 0hrs and 48hrs HZ4 and (B) corresponding band intensities giving ratio of HCN3 to Pol-II. Human SK-NAS neuroblastoma cells were transfected with the HZ4 expression construct and were cultured for 0hrs or 48hrs before RNA extraction occurred. * = $P < 0.05$. (n=3).

7.5 Discussion

One of the major hurdles standing in the way of epilepsy treatment and management, is to uncover the underlying molecular mechanisms and factors which can govern the cellular response to a stress or challenge, that can lead to a seizure, or which can modulate cellular response to future challenges. The NRSF TFs are an intriguing group, linked to epilepsy via their modulation during KA treatment (Palm *et al*., 1998; Spencer *et al*., 2006), and their regulatory roles over a set of genes implicated in epilepsy, including BDNF, TAC1 and NKB (Timmusk *et al.,* 1999; Tabuchi *et al.,* 2002a; Quinn *et al.,* 2002; Spencer *et al.,* 2006; Greco *et al.,* 2007; Howard *et al.,* 2008; Reddy *et al.,* 2009; Chapter 3). NRSF has a well established ability to modulate chromatin structure (Reviewed in Ooi & Wood., 2007), and is known to modulate activity dependent gene expression in neurons (Hara *et al*., 2009) suggesting that this molecule can respond quickly to challenges and has the ability to govern medium to long-term changes in gene expression profiles, in turn modifying the cellular phenotype. NRSF can be incorporated into many of the current hypotheses associated with epilepsy, including a role for adult neurogenesis in the subventricular zone (SVZ) (Gonzalez-Martinez *et al*., 2007; Parent & Murphy., 2008) as NRSF is known to affect adult stem cell lineage (Reviewed by Ballas & Mandel., 2005).

The data presented in this chapter supports a role for NRSF variants modulating many genes which are associated with epilepsy and can therefore be positioned as an early step in the cascade of changes in the CNS that lead to epilepsy. Importantly I demonstrate that the different isoforms of NRSF can direct differential gene expression patterns, in a time dependent manner. For example, the more prolonged over-expression

of REEX1 (48hrs vs 24hrs), led to an increase in the number of genes uniquely modulated by REEX1 (Figure 7.1), with genes belonging to the 'Adaptor/Regulator' group, exhibiting the biggest change (Figure 7.2). Our group and others have shown that NRSF isoforms are differentially modulated in rodent models of SSSE (Palm *et al*., 1998; Spencer *et al*., 2006), therefore the temporal control of these isoforms may have important consequences for the progression of epilepsy. Consistent with this model, are the findings that both full-length NRSF and its truncated isoform sNRSF are differentially modulated by the ACDs CBZ, PHY and LMT, as discussed in Chapter 5.

The over-expression of the NRSF variants was found to have a profound effect on global gene expression patterns, with over 1000 genes modulated at both 24hrs and 48hrs time points, when compared to 0hrs baseline time point controls. It is reasonable to assume that these genes are not all regulated directly by the NRSF variants, but may also be modulated indirectly. However, the number of potential NRSF targets has been proposed to be more that 1000 genes based on the bioinformatic identification of some 1,892 NRSEs in the human genome (Bruce *et al*., 2004), and more recently, the discovery of NRSF binding strongly to 1947 positions in the human genome, using a large-scale ChIP assay (ChIPSeq) (Johnson *et al*, 2007). An important investigation would be to compare our microarray data against the gene lists generated by such genome-wide studies, to determine how many of the genes modulated here, are potential direct NRSF targets, due to the presence of an NRSE or known NRSF binding to the locus of each gene promoter. This analysis is currently being undertaken in collaboration with bioinformatic groups.

The microarray dataset provided a multitude of gene expression changes which would be consistent with a model in which NRSF isoforms are up-regulated as an early response to seizure, with this change in NRSF isoform expression driving a change in expression of numerous genes, directly or indirectly, which have known associations with epilepsy. Genes within well-established epilepsy pathways are found to be modulated in the microarray dataset, consistent with changes one may expect following seizure. Members of the excitatory glutamate system, the glutamate receptors mGluR8 and GRIN2D were found to be elevated in response to both REEX1 and HZ4 over-expression, respectively (Table 7.1 & Table 7.3). Concurringly, repression of the inhibitory GABAnergic system was also observed, notably a reduction in expression of the GABA receptor genes GABBR1 and GABRE was found following REEX1 over-expression (Table 7.2). Furthermore, the microarray indicated that over-expression of REEX1 resulted in the decreased expression of the anti-convulsant neuropeptide Galanin (Table 7.2), and the increased expression of the NK1R, the receptor for the pro-convulsant neuropeptides SP and NKB. Taken together, such changes could lead to a hyper-excitable cellular phenotype, more prone to abnormal neuronal firing.

In addition, changes in ion channel expression were observed, which are consistent with published modulation during or following seizure, such as the up-regulation of the calcium channel CACNB2 following HZ4 over-expression (Table 7.3), which is consistent with previous reports of CACNB2 elevation following seizure (Gastaldi *et al.,* 1998), and a repression of the potassium channel HCN2 following REEX1 over-expression, which is consistent with the observed reduction in HCN2 in KA seizure models (Powell *et al.,* 2008). Finally, the NADH-binding co-repressor CtBP

was also found to be up-regulated by REEX1 over-expression (Table 7.1). This is of particular significance as the mechanisms underlying the anti-epileptic ketogenic diet have recently been eluded to, with NRSF and the NADH-binding co-repressor CtBP, shown to be of critical importance in coordinating a metabolic regulation of the neuronal genes BDNF and its receptor TrkB (Garriga-Canut *et al.,* 2006), which was also found to be modulated by REEX1 in this study (Table 7.1).

Intriguingly, a number of genes known to be modulated by NRSF variants and which are correlated with epilepsy, were notably absent from the dataset including BDNF, TAC1, NKB and SCN2a.. These genes are covered by the Affymetrix microarray employed here, indeed the reported increase in BDNF expression following VPA treatment, was discovered employing the same U133 chip (Fukuchi *et al*., 2009). Therefore the absence of these genes was of interest, and it became important to validate the microarray, to give confidence in the dataset. The modulation of three ion channels was validated via RT-PCR, these being SCN9a (Figure 7.4), HCN2 (Figure 7.5) and HCN3 (Figure 7.6). All three genes were found to be modulated consistent with that reported in the microarray, giving confidence in the dataset. Furthermore, the observed changes in HCN2 expression following REEX1 over-expression, supported previously published reports that HCN2 is repressed by NRSF (Kuwahara *et al.,* 2003), based on the presence of a putative NRSE (Johnson *et al.,* 2007), and gave further confidence in the microarray data. It is possible that the absence of BDNF, TAC1, NKB and SCN2a from the microarray dataset, is due to the lack of regulation of these genes by the NRSF variants at the given time points. Spencer *et al*., revealed that NRSF variant modulation of TAC1 is time dependent, with elevation of these isoforms and TAC1 mRNA found

after 3hrs KA treatment, but not at 24hrs KA treatment (Spencer *et al*., 2006). Furthermore, in Chapter 3, the observed increase in endogenous NKB expression following NRSF variant over-expression was found to be time-dependent (See chapter 3, Figure 3.5). Therefore it is possible that the absence of these genes from the dataset is due to the time courses studied, and therefore it would be of particular interest to monitor the expression of these genes with different, perhaps shorter, time points.

To summarise, I set out to explore the potential role of NRSF isoforms in modulating global changes in gene expression patterns, in a cell line model which was consistent, in part, with the underlying changes observed following seizure (i.e. elevated NRSF isoform expression, following seizure, with the truncated isoform absent in unstimulated cells). Our model is that NRSF isoforms respond to the initial insult or seizure, and this change in NRSF isoform expression can bring about more long-term changes in global gene expression profiles due to NRSF's ability to modify chromatin structure. This could perhaps lead to a more sensitive or hyper-excitable cellular phenotype, more prone to abnormal neuronal firing. The microarray data gives support to such a model as dynamic differential modulation of global gene expression profiles were observed following over-expression of the NRSF variants. These changes in gene expression included a number of genes with known or proposed associations with epilepsy, and notable changes which are consistent with our model included the elevation of members of the excitatory glutamate system and the pro-convulsant receptor NK1R, as well as the repression of some members of the GABAnergic systems and the reduction of the anti-convulsant galanin. Taken together, I propose that NRSF is

key to understanding the molecular mechanisms underlying seizure progression, and as such warrants increased focus in epilepsy research.

CHAPTER 8: Modulation of the NRSF and USF families following cocaine treatment

8.1 Introduction

Throughout this thesis, the role of two transcriptional regulatory systems has been explored with regards to epilepsy. More specifically, the roles of the NRSF and USF TFs in the regulation of two pro-convulsant neuropeptides (NKB and TAC1) have been investigated, as have the response of these TFs to ACD treatment. Furthermore, NRSF isoform over-expression was found to modulate the expression of a multitude of genes, with known or proposed functions in seizure generation and progression. The importance of these regulatory systems in this neurological disorder, have thus become increasingly more apparent throughout this thesis.

It has been known for some time now that seizures can be induced through chemical means, such as KA administration which is routinely used in drug-induced seizure models. There is now an increasing body of evidence to suggest that cocaine administration can also induce seizures, with cocaine-induced seizures reported in both humans and rodents (Lowenstein *et al*., 1987; Hanson *et al*., 1999; Lason., 2001). Furthermore, cocaine's affect on the dopaminergic system is well documented, and recent studies have shown that loss of certain dopamine receptors can induced spontaneous seizures (D1 receptor) (Gantois *et al*., 2007) and enhance susceptibility to pharmacological induced seizures (D2 and D4 receptors) (Weinshenker & Szot., 2002). Taken together, these reports suggest that cocaine can modulate pro-convulsant pathways.

To date, the effect of cocaine treatment on the NRSF regulatory system has yet to be clarified, despite a body of evidence pointing to a correlation. Firstly, cocaine is known to induce the expression of CART (Douglass *et al*., 1995), a transcript which has recently been shown to be repressed by NRSF, via direct binding to a NRSE within the CART promoter (Li *et al*., 2008). Interestingly CART has been shown to be expressed in the hippocampus, an area of the brain important in seizure (Wasterlain *et al*., 2002), and has been shown to co-localise with SP in rodent nuclear accumbens (Hubert & Kuhar., 2005), indicating an overlap with the NRSF regulatory system and CART expression. Cocaine is also known to modulate the expression of a second NRSF-regulated gene; BDNF, with increased BDNF expression observed in the hippocampus (Filip *et al*., 2006), striatum (Zhang *et al*., 2002), amygdala and nucleus accumbens (Grimms *et al*., 2003), following cocaine treatment. Furthermore, cocaine has been shown to up-regulate the expression of the tachykinin receptor NK1R (Renthal *et al*., 2007), a gene also found to be modulated following NRSF isoform over-expression (Chapter 7).

These findings suggest a correlation between cocaine, NRSF and epilepsy. In this chapter, I set out to explore the impact of cocaine treatment on the NRSF regulatory system, in terms of both NRSF isoform expression, and NRSF binding ability, as a potential mechanism underlying cocaine-induced seizures. In addition, I explored the impact of cocaine treatment upon the pro-convulsant tachykinin NKB, and the USF regulatory system, to further explore the hypothesised association of cocaine and these TF systems, and how this could be correlated with epilepsy.

8.2 Aims

- To clarify the affect of cocaine treatment on CART expression in human SH-SY5Y neuroblastoma cells.
- To determine if any cocaine-mediated change in CART expression, arise due to cocaine modulation of NRSF isoform expression.
- To determine if cocaine modulates NRSF binding to the region encompassing the CART NRSE and other putative and determined NRSE-containing regions.
- To explore any impact of cocaine treatment on the expression of the tachykinin NKB and the TFs USF1 and USF2

8.3 Methods

8.3.1 Cell culture and treatment

Human SH-SY5Y cells were used throughout as the cell-line model, since RT-PCR revealed they express both NRSF isoforms (see chapter 3, Figure 3.1), and as SH-SY5Y are considered to be a dopaminergic neuroblastoma cell line (Biedler *et al*., 1978). SH-SY5Y were cultured as outlined in methods section 2.2.2.1.1 and treated as described in section 2.2.2.2. For mRNA analysis, cells were then harvested as detailed in methods section 2.2.3.1, 2.2.3.3 and expression was analysed as described in section 2.2.3.5. For ChIP assays, cells were fixed and protein/DNA interactions cross-linked as described in section 2.5.1.

8.3.2 mRNA expression analysis

8.3.2.1 RT-PCR

The expression of CART, NKB and the house-keeping gene β-actin, was investigated using RT-PCR as described in methods section 2.2.3.4, using the PCR primers set out in Table 2.2.1. Thermal cycle conditions for all were as follows: initial denaturation: 95^{o}C for 2mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds, annealing 64^{o}C for 30 seconds and elongation: 72^{o}C for 1min; and a final elongation step of 72^{o}C for 2mins.

8.3.2.2 Q-PCR

The expression of the NRSF, sNRSF, USF1, USF2 and the house-keeping gene Pol-II, was investigated using qPCR as described in methods section 2.2.3.5, using the

PCR primers set out in Table 2.2.1. CART was not analysed using qPCR as the product size was deemed too large for optimal qPCR conditions, whilst conditions for NKB could not be optimised in time, for qPCR. Thermal cycle conditions for NRSF, USF1, USF2 and RNA polymerase II, were as follows: initial denaturation: 95^{o}C for 3mins for 1 cycle; followed by 40 cycles of denaturation: 95^{o}C for 30 seconds and annealing 62^{o}C for 30 seconds; and a final elongation step of 72^{O}C for 2mins. For sNRSF the annealing temperature was reduced to 58.5^{o}C, with all other conditions remaining the same. Specificity of products was determined by subsequent melt curve analysis from 55°C to 95°C increasing in 0.5°C increments.

8.3.3 TF binding study – ChIP assay

To investigate the impact of cocaine treatment upon NRSF binding to target regions, ChIP was employed as outlined in method section 2.2.5, using the antibodies given in Table 2.3 and the PCR primers given in Table 2.4.

8.4 Results

8.4.1 Cocaine up-regulates CART mRNA expression

CART was originally discovered as a transcript markedly up-regulated following cocaine treatment (Douglass *et al*., 1995). To determine if cocaine treatment affected the NRSF or USF TF family members, it was important to first establish an appropriate cell line model for experimental use. Human SH-SY5Y cells, which have been used in chapters 5 and 6 to explore the impact of ACD treatment upon the NRSF and USF families, were utilised due to their expression of both NRSF isoforms and USF1 and USF2. CART expression was observed in vehicle control treated SH-SY5Y cells, and CART mRNA was found to be significantly elevated following 4hrs cocaine treatment (at both 1μM and 10μM) compared to vehicle controls ($P = < 0.05$) (Figure 8.1) (n=5). The more prolonged 24hrs cocaine treatment had no impact on CART mRNA expression compared to vehicle controls. ($P > 0.05$) (Figure 8.1) (n=5). Thus cocaine induces CART mRNA expression in a time-dependent manner, in human SH-SY5Y cells.

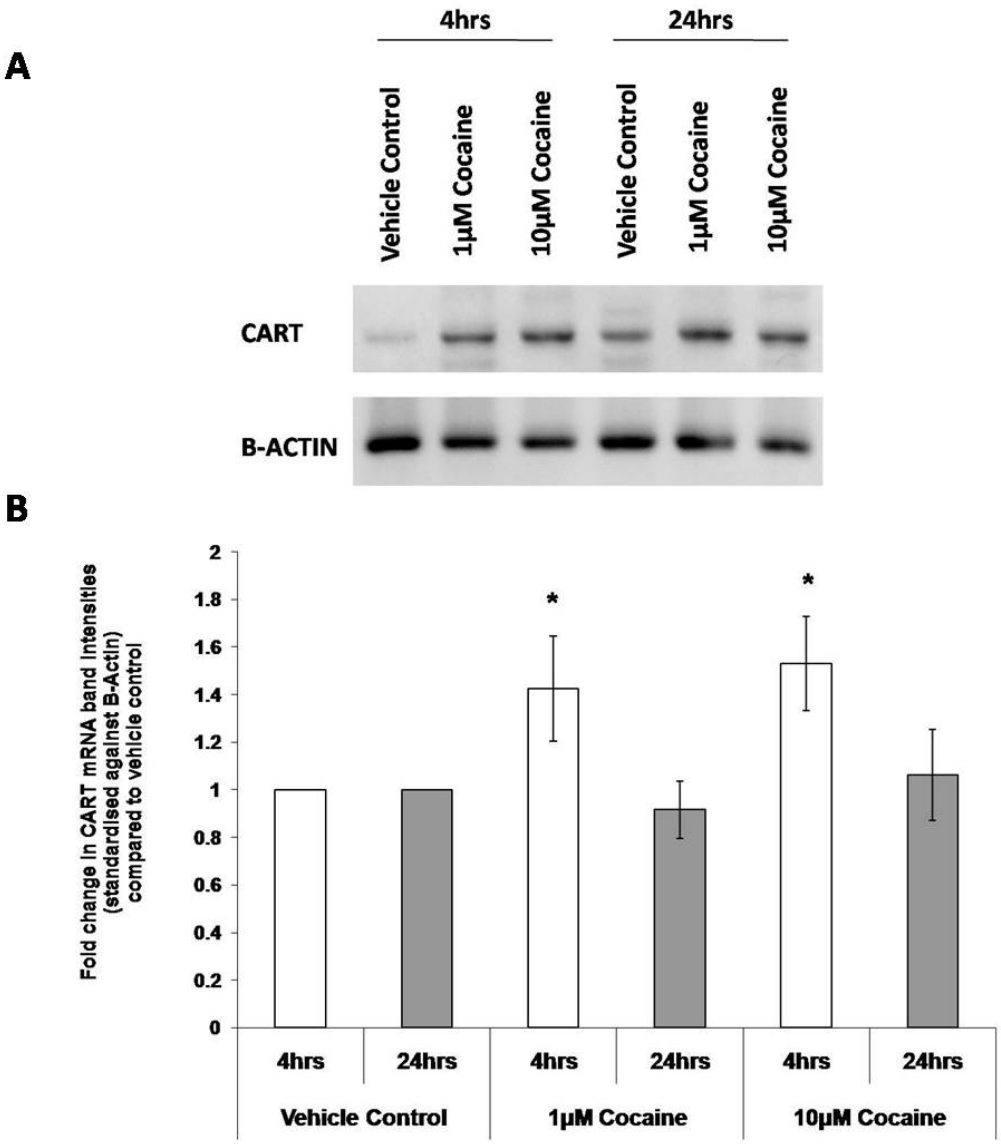

Figure 8.1 Modulation of CART mRNA expression following cocaine treatment. RT-PCR analysis of CART mRNA expression in response to cocaine treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 1µM or 10µM cocaine, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. (A) Representative mRNA expression of CART. Changes to endogenous CART mRNA expression was analysed using RT-PCR, with band intensities normalised against the house keeping gene β-actin, and (B) mean fold changes compared to vehicle control given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$. (n=5).

8.4.2 NRSF isoform expression is modulated following cocaine treatment

Full-length NRSF has previously been shown to repress the expression of CART (Li *et al*., 2008), and whilst cocaine's impact on CART is well documented, its impact on NRSF expression has yet to be determined. I reveal for the first time that cocaine treatment causes a marked reduction in the mRNA expression of both full length NRSF (Figure 8.2a) and the truncated isoform sNRSF (Figure 8.2b). 4hrs cocaine treatment (both 1µm and 10µM) caused a significant 50% reduction in NRSF mRNA expression compared to vehicle controls ($P = < 0.001$) (Figure 8.2a) (n=3), which is consistent with the observed elevation of CART expression, as one may expect. In addition 24hrs 1µM cocaine also caused a less significant decrease in NRSF expression ($P = < 0.05$) (Figure 8.2a) (n=3). In addition, the truncated NRSF isoform (sNRSF) was also found to be reduced following cocaine treatment, in a duration and concentration dependent manner, with only 4hrs 10µM cocaine treatment invoking a significant ~30% reduction in sNRSF expression, compared to vehicle controls ($P = < 0.05$) (Figure 8.2b) (n=3).

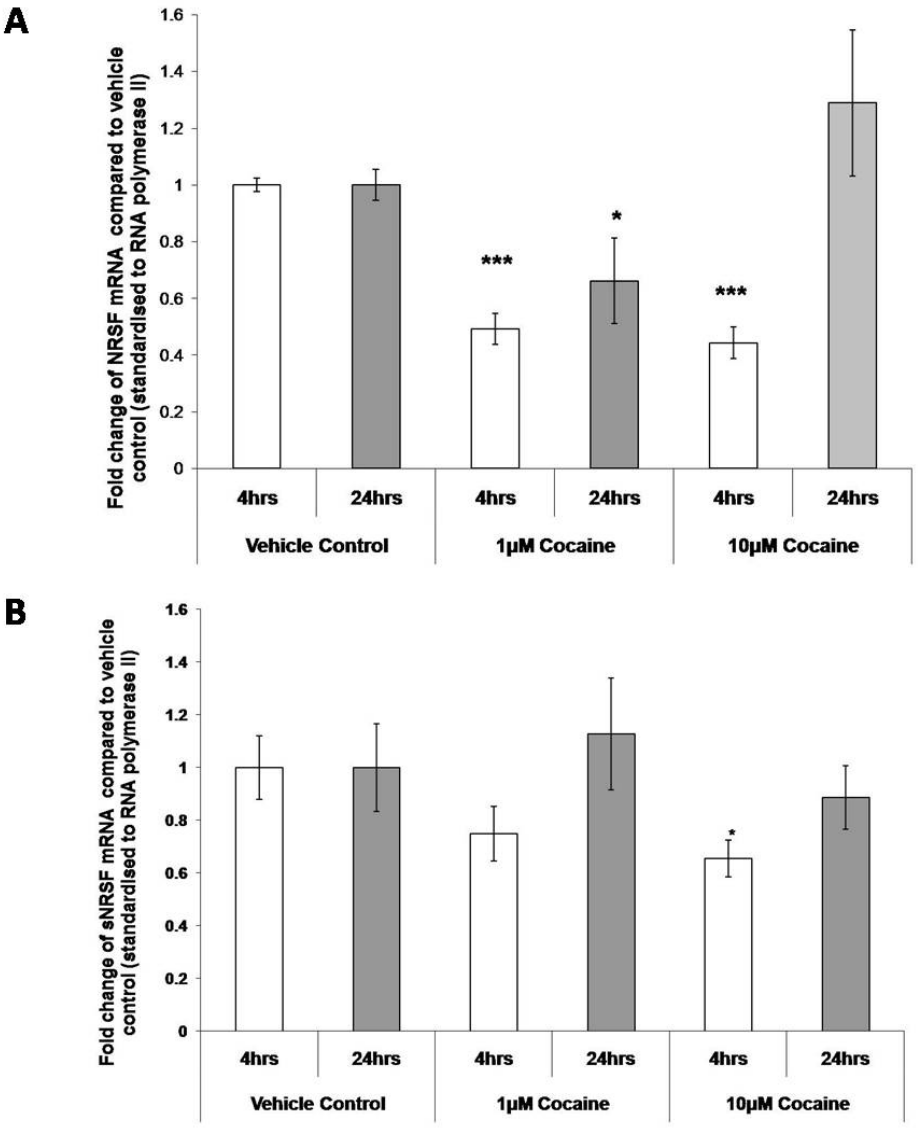

Figure 8.2 Modulation of NRSF isoforms following cocaine treatment. qPCR analysis of **(A)** NRSF and **(B)** sNRSF mRNA expression in response to cocaine treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 1µM or 10µM cocaine, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of NRSF and the truncated isoform sNRSF, were analysed using qPCR, normalised against Pol-II, mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$ & *** = $P = < 0.001$. (n=3).

8.4.4 Cocaine may affect NRSF binding to target NRSE-containing regions

Li *et al*, showed that NRSF binds to a NRSE within the CART promoter region, repressing CART expression (Li *et al*., 2008). I have shown that 4hrs 1μM and 10μM cocaine treatment results in an increase in CART expression (Figure 8.1) and a corresponding decrease in NRSF mRNA expression (Figure 8.2). To determine if this reduction in NRSF could affect CART expression directly, I undertook preliminary investigations to explore the impact of 4hrs 10μM cocaine on NRSF binding to the CART promoter, using a ChIP assay. NRSF was shown to be capable of binding to the CART NRSE-containing promoter region in SH-SY5Y cells in chapter 5 (see Figure 5.14). Here I show that NRSF is also bound to the CART promoter region in vehicle control treated SH-SY5Y cells, and that NRSF binding to this region may be reduced following 4hrs 10μM cocaine treatment, when normalised to the non-specific background (negative) control; IgG (Figure 8.3). However, as the IgG band is found to be particularly bold, it is possible that cocaine is affecting only the IgG background binding. Thus one cannot conclude NRSF binding to the CART promoter is affected by cocaine at present, and further investigations are required. Due to the proposed importance of cocaine in epilepsy, I explored the impact of cocaine on NRSF binding to the region containing the BDNF NRSE and the NKB and TAC1 promoter regions. In preliminary findings, 4hrs 10μM cocaine treatment was found to reduce NRSF binding to all three NRSE-containing regionss, compared to vehicle controls (Figure 8.3). This may be consistent with the fact that BDNF is up-regulated following cocaine treatment (Zhang *et al.,* 2002; Grimm *et al.,* 2003; Filip *et al*., 2006), as reduced binding would

inhibit NRSF mediated repression of BDNF expression, and hence BDNF expression would be induced.

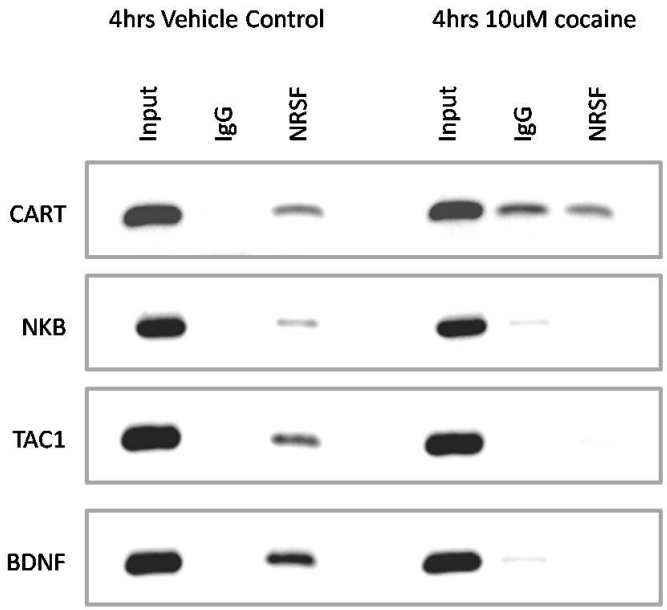

Figure 8.3. Modulation of NRSF binding to target NRSE-containing regions following 4hrs cocaine treatment in human SH-SY5Y cells. ChIP analysis of NRSF binding to characterised or putative NRSE-containing regions in SH-SY5Y cells treated with either 4hrs 10μM cocaine or vehicle control. IgG was included as a control for non-specific background binding. NRSF binding to the NKB, TAC1and BDNF NRSE-containing regions was found to diminished following cocaine treatment when compared to vehicle control.(n=1).

8.4.5 Endogenous NKB expression is unaffected following cocaine treatment

The potential reduction of NRSF binding to both NKB and TAC1 promoter regions following cocaine treatment, may suggest the loss of NRSF-mediated activation of these two genes. Consequently I sought to explore the impact of cocaine treatment on the expression of these neuropeptides. As NKB (and not TAC1) was expressed in SH-SY5Y cells, I focused on cocaine modulation of endogenous NKB expression.

Intriguingly neither 4hrs nor 24hrs (1µM or 10µM) cocaine treatment had any significant impact upon endogenous NKB expression when compared to vehicle controls (*P = > 0.05*) (Figure 8.4) (n=5).

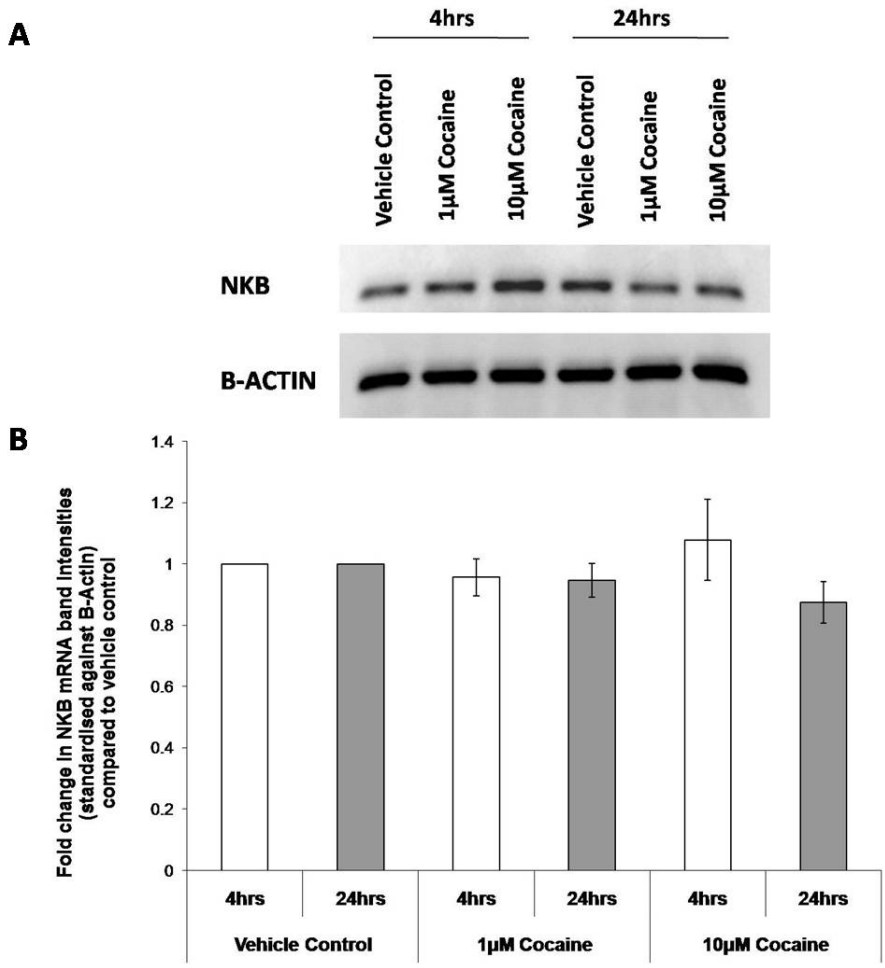

Figure 8.4 Modulation of NKB mRNA expression following cocaine treatment. Reverse transcriptase-PCR (RT-PCR) analysis of NKB mRNA expression in response to cocaine treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 1µM or 10µM cocaine, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. (A) Representative mRNA expression of NKB. Changes to endogenous NKB mRNA expression was analysed using RT-PCR, with band intensities normalised against the house keeping gene B-Actin, and (B) mean fold changes compared to vehicle control given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = *P = < 0.05*. (n=5).

8.4.6 Cocaine represses USF1 and USF2 mRNA expression

The findings that cocaine treatment had no impact upon NKB expression, whilst abolishing NRSF binding to the putative NKB NRSE, were unexpected. The USF TFs were shown in chapter 3 to act as repressors of NKB proximal promoter activity, and thus I was interested in exploring the possibility that cocaine may modulate USF gene expression. Human SH-SY5Y cells were treated with either 1μM or 10μM cocaine, or vehicle control, for either 4hrs or 24hrs. The response of both USF1 and USF2, to cocaine was found to be virtually identical. 4hrs cocaine treatment (both 1μM and 10μM) induced a significant reduction in both USF1 (Figure 8.5a) and USF2 (Figure 8.5b) mRNA expression ($P = < 0.05$) (n=3). Furthermore, 24hrs 10μM cocaine treatment also induced a reduction in both USF1 and USF2 (Figure 8.5a & Figure 8.5b, respectively) ($P = < 0.05$) (n=3). Therefore, cocaine treatment not only reduces the expression of the NKB-activator NRSF, but also the NKB-repressors USF1 and USF2, and this may explain why NKB is unaffected following cocaine treatment.

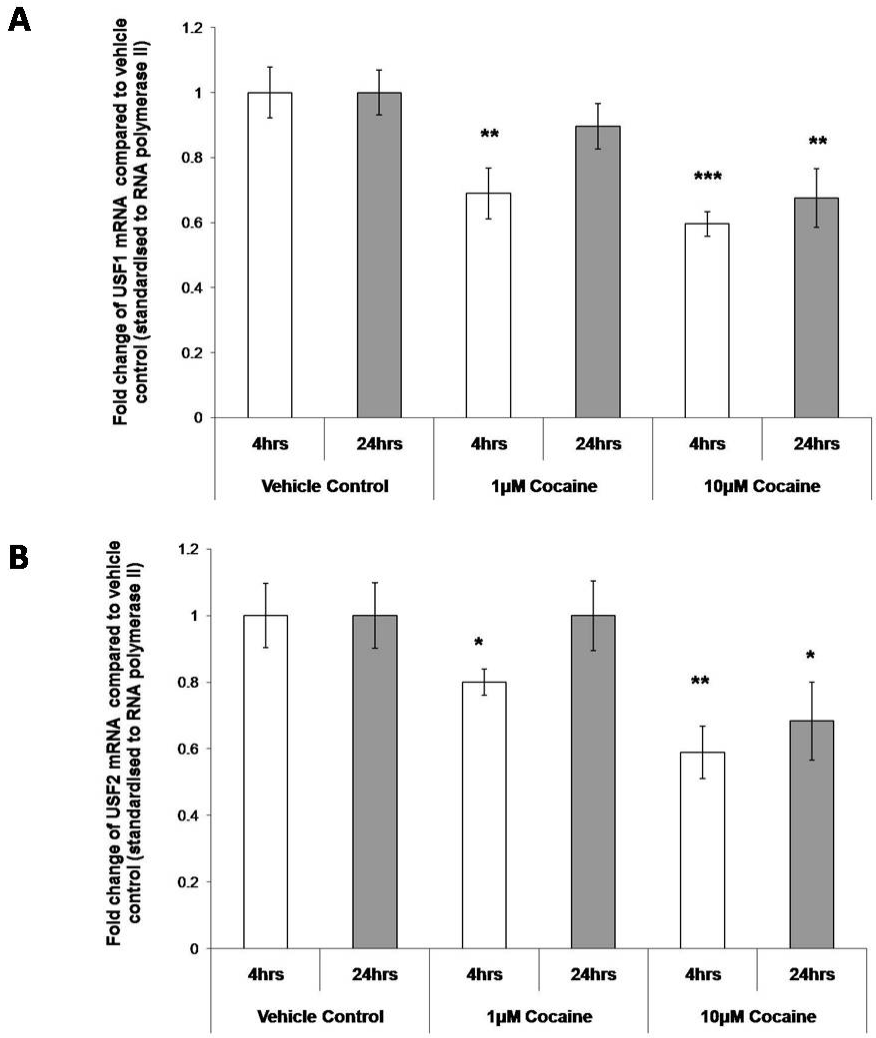

Figure 8.5 Modulation of USF1 and USF2 following cocaine treatment. Quantitative-PCR (qPCR) analysis of **(A)** USF1 and **(B)** USF2 mRNA expression in response to cocaine treatment in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with either 1µM or 10µM cocaine, or vehicle control, for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of USF1 and USF2 was analysed using qPCR, normalised against the house keeping gene RNA polymerase II (Pol-2), mean fold changes compared to vehicle controls are given. Standard errors are given as y-axis error bars. Students *t*-test was used to compare fold changes against vehicle controls with: * = $P = < 0.05$, ** = $P = < 0.01$ & *** = $P = < 0.001$. (n=3).

8.5 Discussion

In the previous chapters, the NRSF isoforms and the USF family of TFs have been shown to govern the regulation of the NKB and SP encoding genes, TAC3 and TAC1, respectively. These TFs were later found to be modulated by ACD treatment, and their roles (specifically the NRSF isoforms) in modulating epilepsy-associated gene expression, was highlighted following their over-expression. This thesis has provided evidence supporting the hypothesis that these TFs are important in modulating gene expression in relation to seizure. To further explore such a hypothesis, I explored the response of these TFs to cocaine, a drug which has been reported to induce seizures (Lowenstein *et al*., 1987; Hanson *et al*., 1999; Lason., 2001), and is known to modulate the dopaminergic system, a system also implicated in seizures generation and progression (Gantois *et al*., 2007; Weinshenker & Szot., 2002; Witkin *et al*., 1998).

Cocaine is known to induce the expression of the NRSF-regulated genes CART (Douglass *et al*., 1995; Li *et al*., 2008) and BDNF (Filip *et al*., 2006; Zhang *et al*., 2002; Grimms *et al.,* 2003; Timmusk *et al*., 1998; Tabuchi *et al*,. 2002), however despite this, the effect of cocaine on NRSF has yet to be explored. Here, I demonstate that cocaine treatment induces a significant repression of both full-length and truncated NRSF isoform expression (Figure 8.2). The observed repression of NRSF following cocaine treatment coincided, in part, with an observed increase in CART mRNA expression (Figure 8.1), which would be consistent with the findings that NRSF represses CART expression (Li *et al*., 2008), whilst cocaine increases CART expression (Douglass *et al.,* 1995). Furthermore, cocaine is known to induce BDNF expression, and our findings

suggest that this could be through cocaine's repression of NRSF, which in turn would lead to de-repression of BDNF, although this has yet to be clarified.

How cocaine induces this observed reduction in NRSF expression, has yet to be determined. One potential mechanism could be through the up-regulation of the methyl-CpG-binding protein MeCP2. Cassel *et al*, demonstrated that cocaine administration in rats led to a significant up-regulation of MeCP2 (Cassel *et al*., 2006) and interestingly, MeCP2 has been shown to bind to the NRSF promoter, and was proposed to function in NRSF repression based upon the observed up-regulation of NRSF in MeCP2 knock-out mice (Abuhatzira *et al*., 2007). Therefore, cocaine may enhance MeCP2 expression, which could lead to repression of NRSF and in turn, enhanced expression of CART and BDNF. In support of this, MeCP2 deficiency has been correlated with decreased BDNF expression, due to enhanced repression of the BDNF promoter, resulting from elevated NRSF levels (Abuhatzira *et al*., 2007). It would therefore be of interest to explore the impact of cocaine upon MeCP2 binding to the NRSF promoter, to clarify if cocaine-mediated MeCP2 repression of NRSF occurs.

As aforementioned, NRSF is known to be up-regulated during seizure (Palm *et al*., 1998; Spencer *et al*., 2006), thus the cocaine-mediated reduction in NRSF expression, appears to be contradictory to a hypothesis suggesting cocaine-induced seizures operates via modulation of the NRSF regulatory system. However, we have previously postulated that it is the ratio between full-length NRSF and the truncated isoforms which is the key factor in governing seizure generation and progression (Spencer *et al*., 2006). Here I find that cocaine has a greater impact upon full-length NRSF expression compared to sNRSF expression, with a highly significant 60%

reduction of NRSF observed compared to a 30% reduction in sNRSF (Figure 8.2). Thus cocaine treatment modulates the NRSF isoforms differentially, which would impact upon the NRSF isoform ratio, elevating the levels of the truncated isoform compared to full-length NRSF. This swing in the ratio towards the truncated isoform, is consistent, in part, with that observed in rodent seizure models (Spencer *et al*., 2006), suggesting that this may be an underlying mechanism orchestrating cocaine-induced seizures.

The data presented here demonstrating that cocaine modulates NRSF expression, a TF with a well established role in chromatin remodelling (reviewed in Ooi & Wood., 2007), may suggest such a role in epigenetics for cocaine. In support of this, cocaine has been shown to induce hyperacetylation of histones at the cFos, BDNF and Cdk5 promoters, coinciding with elevated expression of these three genes (Kumar *et al*., 2005). As a consequence, I was interested in exploring the impact of cocaine treatment upon NRSF binding to target NRSEs within the neuropeptides CART, NKB and TAC1, and the neurotrophic factor BDNF.

One may expect that as cocaine induces CART expression (Figure 8.1), and NRSF represses CART, that cocaine would not only reduce NRSF expression (as seen in Figure 8.2), but may also impair NRSF binding to the CART NRSE, thus leading to de-repression. Unfortunately the preliminary ChIP assay presented here is inconclusive with regards to cocaine's impact upon NRSF binding to the CART promoter, and further experimentation is required to provide an answer. (Figure 8.3). Interestingly, cocaine was found to reduce NRSF binding to the TAC1 and NKB promoter regions and the region encompassing the BDNF NRSE (Figure 8.3). This may well be a direct consequence of diminished NRSF expression following cocaine treatment, or it may be

due to an inhibition of a mechanism governing NRSF binding. Such a mechanism could be a common binding partner, which coordinates NRSF binding to such NRSEs. I have previously alluded to a shared regulatory role of NKB, TAC1 and BDNF between the NRSF and USF TFs, and I reveal here that cocaine reduces the expression of both USF1 and USF2 in this cell line model (Figure 8.5). Perhaps USF proteins help to coordinate NRSF binding, and reduced expression of USF1 or USF2 may inhibit NRSF recruitment to target NRSEs. It would be of great interest therefore in future to characterise USF binding to these genes in response to cocaine treatment, particularly NKB, TAC1 and BDNF. Furthermore, as NRSF repression of CART was found to be HDAC dependent (Li *et al*., 2008), it would be of interest to characterise HDAC recruitment to these target NRSEs to gain a greater understanding of how cocaine modulates the chromatin structure and in turn, expression of these genes.

The data presented here, and by Li *et al*, indicates that CART expression is regulated by NRSF binding to the CART NRSE, and that cocaine treatment not only reduces NRSF expression, but also abolishes NRSF binding to the CART NRSE, with the net result that cocaine induces CART expression. The ChIP assay also suggested that cocaine reduces NRSF binding to the BDNF NRSE-containing region, which would be consistent with the reported elevated in BDNF following cocaine treatment (Zhang *et al.,* 2002; Grimm *et al.,* 2003; Filip *et al*., 2006), as like CART, BDNF is repressed by NRSF (Timmusk *et al*., 1999). NRSF binding to the TAC1 and NKB promoter region was also found to be abolished following cocaine treatment in preliminary findings, and thus I wished to explore the impact of cocaine on the expression of these neuropeptides. As the cell line utilised only expressed NKB, I focused on endogenous NKB expression,

however in future, the experiment should be repeated in the TAC1-expressing SK-N-AS cell line, to gain data on cocaine's affect on TAC1 expression.

Intriguingly, endogenous NKB expression was found to be unaffected by the cocaine treatment employed here (Figure 8.4).A plausible reason for this is the observed down-regulation of USF 1 and USF2 expression (Figure 8.5). In chapter 3, I revealed that USF repressed the activity of the NKB promoter, suggesting that USF repressed NKB expression. With the cocaine-induced reduction of both NRSF isoform and USF1 and USF2 expression, both the NKB-activator and repressor are reduced, and so the ratio between the two is maintained to some degree, resulting in limited change in NKB expression. This is similar to the observations that CBZ treatment impaired both NRSF and USF regulation of the NKB promoter, resulting in only a limited change in NKD expression (see chapter 3). This provides further support for a shared regulatory role of NKB for both NRSF and USF TF families, as discussed in chapter 3.

Throughout this thesis, I have discussed the apparent importance of both the NRSF and the USF regulatory systems in governing gene expression, with regards to seizure. The relationship between cocaine and seizures has become increasingly apparent, and the data presented in this chapter suggests that cocaine-induced seizures may arise due to cocaine's affect upon these two TF systems. More work is required in this field to unravel the underlying molecular mechanisms at work which initiate and propagate seizures, and perhaps comparing the affects of cocaine administration with more classical pro-convulsant (i.e. KA) or even anti-convulsant treatment, may help to reveal the fundamental mechanisms, and may hopefully present new therapeutic targets for anti-epileptic treatment.

CHAPTER 9: NRSF and USF TFs regulate pro-convulsant neuropeptides and are modulated by ACD treatment.

The underlying aim of this thesis was to explore and gain a greater understanding of the molecular changes which may be relevant to the progression of epilepsy. It is generally accepted that the epilepsy condition can arise from an initial insult, such as a brain trauma, and can result, over time, in recurrent spontaneous seizures. The phrase 'over time' is especially relevant, with a well documented latent period existing between the initial insult and the epilepsy condition proper. It is believed that during this latent period, major changes occur in the brain, at both the cellular (e.g. neuronal circuitry reorganisation) and molecular level (e.g. gene expression changes), in a process known as epileptogenesis. This thesis was focused on molecular changes relevant to epileptogenesis, particularly that of neuropeptide gene regulation. In addition to immediate changes in gene expression, the process of chromatin remodelling is a strong candidate for orchestrating molecular changes during epileptogenesis. Chromatin remodelling not only induces widespread changes in gene expression (i.e. multiple genes affected) but can induce medium to long-term changes in gene expression patterns, which would affect the long-term phenotype of the cell.

Support for the importance of chromatin remodelling or epigenetic changes, in epileptogenesis has arrived from multiple sources. Firstly, one of most affective ACDs prescribed, VPA, is an inhibitor of HDAC activity. This HDAC inhibitor activity suggests that VPA prevents the repression of certain genes, which in turn implies that HDAC-mediated gene repression is a key factor in seizure generation and progression. In addition to this, a number of groups have shown that histone modifications occur

following SE, affecting the expression of certain genes. These include histone H4 acetylation of the BDNF promoter, inducing elevated BDNF mRNA expression (Tsankova *et al*., 2004) and histone deactylation at the glutamate receptor (GluR2) promoter, repressing GluR2 expression (Sanchez *et al*., 2001; Jia *et al*., 2006). Furthermore, TFs such as NRSF, which have well-documented roles in coordinating epigenetic modifications, are found up-regulated during SE (Palm *et al*., 1998; Spencer *et al*., 2006).

Throughout this thesis I have focused on two distinct TF families; NRSF and USF. Our group has previously postulated that NRSF is a key TF in epileptogenesis based on the fact that it is modulated early during seizure (thus representing an initial target) (Spencer *et al*., 2006) and because NRSF can govern epigenetic changes, thus can bring about long-term changes in chromatin structure, and in turn long term changes in gene expression (Ooi & Wood., 2007). This ability confers to the epileptogenesis model, with molecular changes occurring over time (latent period), in response to an initial insult, which would modify long-term changes in gene expression. In support of this, a number of NRSF-regulated genes are found to be modulated during seizure, including BDNF (Koyama & Ikegaya., 2005), TAC1 (Palm *et al*., 1998; Spencer *et al*., 2006), Snap25, SCG10 and μ-opioid receptor (Becker *et al*., 2002). In addition, USF also has the capacity to induce epigenetic changes, and has been correlated with seizure, with USF1 null mice exhibiting increased susceptibility to spontaneous seizures (Sirito *et al*., 1998). As with NRSF, a number of USF-regulated genes are modulated during seizure including BDNF and TAC1, as well as KCC2 (Markkanen *et al*., 2008) and the $GABA_B$

receptor (Steiger *et al.,* 2004), suggesting that USF may also govern epigenetic changes in response to an initial insult, and thus may be of significance to epileptogenesis.

9.1 Modulation of Neuropeptide expression

Following a seizure, the expression of multiple neuropeptides is altered, with enhanced expression of pro-convulsant neuropeptides SP and NKB (Liu *et al.,* 1999; Liu *et al*., 2000; Marksteiner *et al*., 1992; Sperk *et al*., 1990) and reduced expression of anti-convulsant neuropeptides galanin, dynorphin, somatostatin and NPY observed (Mazarati *et al*., 1998; Mazarati *et al*., 1999; Sperk *et al*., 1986; Sperk *et al.,* 1992). This enhanced ratio of pro-convulsant to anti-convulsant neuropeptide expression, leads to a hyperexcitable neuronal phenotype, with a greater propensity to seizure. In this thesis, I have investigated the mechanisms governing the regulation of the NKB encoding gene, TAC3, in a human neuroblastoma cell line and have further explored those governing the SP-encoding gene TAC1.

I have presented data that indicates that both NRSF and USF TF families have a role in governing the regulation of TAC3. Firstly, I identified a putative NRSE within the TAC3 proximal promoter regions, spanning +50 to +71, in relation to the TSS. Both NRSF and the truncated isoform sNRSF were found to bind to the region encompassing this putative NRSE in preliminary ChIP assays, both acted as activators of NKB promoter activity and both were found to differentially enhance endogenous NKB expression, in a human neuroblastoma cell line. These findings support the theory that both NRSF and its truncated isoform can act as pro-convulsant TFs, with both TFs found to activate the promoter of the pro-convulsant tachykinins NKB and SP (Spencer *et al*., 2006). Intriguingly, NRSF was found to act as an activator of NKB expression, mirroring

its activator roles of two other neuropeptide genes; TAC1 and AVP (Quinn *et al*., 2002; Spencer *et al*., 2006), but is perhaps contradictory to its more established role as a transcription repressor. It is possible that NRSF regulates NKB indirectly, by repressing another factor important in NKB regulation. However the presence of the putative NRSE within the NKB proximal promoter region, together with the preliminary observations that NRSF could bind to the region encompassing the putative NRSE, suggests that NRSF-mediated regulation of NKB is direct. The question therefore remains, why does NRSF activate NKB expression?

One potential explanation may be due to the location of the putative NKB NRSE. As discussed in chapter 3, Bessis *et al*, revealed that the location of the NRSE can confer whether the NRSE acts as an activator element or a repressor element in neuronal cells. NRSEs located within 50bp of the TATA box of a synthetic promoter, were found to act as activator elements, whilst those further upstream acted as repressor elements, in neuronal cells (Bessis *et al*., 1997). The putative NKB NRSE is located +51 from the TSS and so is close to this boundary indicated by Bessis *et al*, suggesting that the NKB putative NRSE could acts as an activator element. This would be consistent therefore with the activation of NKB by NRSF presented here. In addition, other genes shown to be activated by NRSF, have similar NRSE locations, with the rat TAC1 NRSE at -21 to +4 (Quinn *et al*., 2002) and the AVP NRSE -2 to +23 (Coulson *et al*., 1999). Thus the close proximity of these NRSEs to the TSS, may confer transcriptional activator roles, consistent with the early findings of Bessis *et al*.

An alternative explanation may come from the sequence of the putative NKB NRSE. The putative NKB NRSE shares greater homology to the NRSEs of TAC1 (rat)

and AVP (human), both of which have been shown to be activated by NRSF (Quinn *et al*., 2002; Spencer *et al*., 2006; Howard *et al*., 2008; Coulson *et al*., 2000), than to the classical 21bp canonical sequence (see chapter 3, Figure 3.1 and Appendix 5). Recently the classic 21bp canonical NRSE sequence has been shown to be a bipartite sequence, with a left half-site and a right half-site. Interestingly, whilst NRSF can bind to either half-site, individually neither half-site is an effective repressor, with NRSF binding to both half-sites required for repression (Patel *et al*., 2007). Through sequence comparisons, I have revealed that the NKB NRSE shares a great degree of homology to the left-half site (sequence: CAGCACC), exclusively, with little conservation between the right-half site and the NKB (putative) NRSE. This is also seen in the TAC1 sequences (both rat and human) and the AVP sequence (Figure 3.1 and Appendix 5). It is thus plausible that NRSF can recognise and bind to these neuropeptide NRSEs, but as they lack a strong homology to the right-half site, perhaps the binding is not sufficient to induce repression. Intriguingly, I have also shown that the putative NKB NRSE has not one, but two left-half site NRSE sequences, with a second match to the left-half site sequence CAGCACC, discovered within our putative NKB NRSE region (Appendix 5). This is not found in the TAC1 nor the AVP NRSE region, indicating that this is exclusive to the NKB promoter. This doublet may confer different binding properties of NRSF to the NKB putative NRSE, which would be interesting to study further in the future.

In addition to NRSF mediated regulation of NKB, this thesis has also indicated that the USF TFs can also regulate the NKB promoter, repressing NKB proximal promoter activity. A putative E box site was identified, spanning +160 to +166, and USF1 and USF2 were found to bind to the region encompassing this E box in

preliminary ChIP assays, in a cell specific manner. In sNRSF expressing cells (SH-SY5Y) USF2 and not USF1 was found bound to the NKB proximal promoter, whilst in cells which do not express sNRSF (SK-N-AS), USF1 and not USF2 were found bound. This may indicate competition between the USF1 and USF2 for occupancy of the E box site, which may be influenced by the presence of sNRSF.

As well as investigating the regulation of the NKB-encoding TAC3 gene, I sought to further explore the regulation of the SP-encoding TAC1 gene. Previous studies have revealed that USF can regulate a number of NRSF-regulated genes, including BDNF (Tabuchi *et al*., 2002), AVP (Coulson *et al*,. 2003), TAC1 (Paterson *et al*., 1995; Gerrard *et al*., 2005) and now NKB. NRSF mediated regulation of human TAC1 has been shown to operate in synergy with NFκB (Greco *et al*., 2007), and due to the close proximity of the NRSE to a single or multiple E box motifs in the TAC1, AVP and TAC3 promoter regions, I sought to explore a potential synergistic regulatory mechanism between NRSF isoforms and the USF TFs, by utilising the well-characterised rat TAC1 promoter reporter gene constructs.

The experiments undertaken (and presented in chapter 4) suggest that NRSF and USF can work together, driving enhanced rat TAC1 reporter gene activity, compared to when over-expressed individually. The truncated NRSF variant construct (HZ4) was found to activate the rat TAC1 promoter, consistent with previous reports (Spencer *et al*., 2006). This activation was further enhanced when the USF TFs were co-over-expressed with HZ4, suggesting that truncated NRSF can work in synergy with USF1 or USF2 to activate the TAC1 promoter. Interestingly this was found to be dependent on the E box located at -60 in relation to the TSS, as when this was mutated, this synergistic up-

regulation of the rat TAC1 promoter activity was abolished. This E box site is the closest to the rat TAC1 NRSE, suggesting that this close proximity is important in sNRSF-USF synergy. Moreover, it suggests that USF binding to this E box is necessary for this sNRSF-USF synergy, as the mutation should prevent USF binding at that site. In support of this, in cells not expressing sNRSF (SK-N-AS), both USF1 and USF2 were found bound to the human TAC1 proximal promoter, whilst in sNRSF-expressing cells (SH-SY5Y), USF1 was displaced and sNRSF was found bound. This suggests that sNRSF and USF2 (in this case) interact, displacing USF1 from the TAC1 promoter region. However these findings are of a preliminary nature, and repeated investigations are required in the future to better understand the mechanism operating. Furthermore, I have yet to explore an interaction between these TFs, but this would be an interesting future study.

Whilst these findings were obtained in a human neuroblastoma cell line, and not a more complex *in vivo* model, they do provide support for the potential importance of the USF TFs in epileptogenesis. The date indicates that the USF TFs have the capability to modulate the expression of two pro-convulsant neuropeptides, and whilst USF modulation during seizure has yet to be clarified, other bHLH factors are known to be modulated in response to seizure, including Mash1, Id2 and Hes5 (Elliot *et al*., 2001). Furthermore, I have shown that both USF1 and USF2 are up-regulated in response to KA in the SH-SY5Y cell line (chapter 6). If the USF TFs were also up-regulated in response to seizure in an *in vivo* model, this may have a significant impact on the expression of a multitude of genes including BDNF, TAC1 and TAC3. Subsequently, I hypothesise that

the modulation of the USF TFs and target genes, in an *in vivo* SE model is an important future experiment, which may reveal new aspects to SE and epileptogenesis.

9.2 NRSF and USF are targets of ACD treatment

Throughout this thesis, I have proposed that the NRSF and USF TF families are important in epileptogenesis, responding to an initial insult (such as KA stimulation) and governing long term changes in gene expression, particularly governing regulation of pro-convulsant neuropeptides-encoding genes (TAC3 and TAC1). If these TFs were truly important in governing such changes in response to a seizure, then perhaps these TFs would be targeted by ACDs. In this thesis I have shown that both NRSF isoforms, as well as USF1 and USF2 are differentially modulated by ACD treatment, in terms of their mRNA expression and ability to bind to target DNA sequences in a human neuroblastoma cell line model.

The first indication that ACDs could target and modulate the NRSF and USF regulatory systems, came in chapter 3, where CBZ treatment was found to abolish both NRSF and USF mediated regulation of NKB proximal promoter activity. CBZ was found to impair both NRSF variant-mediated activation of the NKB promoter, and USF-mediated repression. This resulted in a modest repression of endogenous NKB mRNA expression, indicating that CBZ could reduce the expression of this pro-convulsant neuropeptide. Following on from this, I presented data showing CBZ could also reduced the expression of the second pro-convulsant neuropeptide, the SP-encoding TAC1 gene, in TAC1-expressing SK-N-AS cells. These findings indicate that CBZ may invoke its anti-convulsant properties through the repression of pro-convulsant neuropeptides, and that the CBZ target, the type II sodium channel, is a potential member of the pathway

which governs both TAC1 and TAC3 gene regulation. Thus modulation of this pathway may have a downstream impact upon pro-convulsant neuropeptide expression, which is of interest for future study.

The expression of the NRSF and USF TFs were found to be differentially modulated following treatment with three different ACDs. The three ACDs chosen, share a common initial target, the type II sodium channel, but have been shown to induce different downstream effects (Rogawski & Loscher., 2004a), and this held true in terms of modulating these TFs. CBZ was found to repress both USF1 and USF2, as well as the truncated sNRSF isoform, which considering the importance of these factors in TAC1 regulation, is consistent with the repression of TAC1 expression. NRSF however was modulated in a treatment duration-dependent manner, with NRSF up-regulated following 4hrs CBZ, whilst down-regulated following 24hrs treatment. PHY had less of an effect on the TFs, at the duration and concentrations tested, with nil effect on the expression of NRSF and USF2. PHY did however cause a reduction in sNRSF and an increase in USF1, which may explain why PHY had little effect on TAC1 expression (Appendix 1), as the reduction of sNRSF, whilst may reduced TAC1 expression, was balanced by an increase in USF1 expression, which may enhance TAC1 expression. LMT was the only drug not to repress sNRSF expression, but did induce an increase in NRSF, whilst invoking a decrease in both USF1 and USF2 expression. Thus each drug induced a different response in terms of NRSF and USF TF expression. This is the first time such ACDs have been shown to modulate the expression of these TFs and could be seen as an important step in further elucidating how these drugs elicit their anti-convulsant properties or a step to delineate specific mechanisms of such drugs.

Consistent with this differential affect upon the NRSF and USF regulatory systems, the ACDs invoked different affects on both NRSF and USF (USF2 in this case) binding to target DNA sequences, albeit in preliminary investigations. CBZ treatment was found to enhance NRSF binding to the Scg10, TAC1 and NKB (putative) NRSEs compared to vehicle controls, whilst PHY reduced NRSF binding to the Scg10 and TAC1 NRSE, and elevated NRSF binding to the L1CAM and NKB (putative) NRSEs, compared to vehicle controls. These findings again represent a possible mechanism of action for these ACDs, suggesting that these ACDs could modulate NRSF binding to target DNA sequences, which may have significant impacts upon the regulation of target genes. CBZ treatment was also found to reduce USF2 binding to both the TAC1 and NKB proximal promoters, perhaps helping to explain CBZ mediated repression of TAC1 mRNA expression, and CBZ induced impairment of USF regulation of the NKB promoter.

Whilst these ACDs had little impact upon the localisation of the NRSF isoforms nor the USF proteins, they did induce significant changes to the endogenous expression of these TFs, thus revealing a novel mechanism of action for these ACDs. Furthermore, as these ACDs target and modulate both the NRSF and USF regulatory systems, the potential importance of these TFs in seizure and epileptogenesis becomes more plausible.

9.3 Is NRSF important in epileptogenesis?

The thesis so far alludes to the importance of the NRSF and USF TF families in seizure and epileptogenesis, and I have previously stated that a potential mechanism at work during epileptogenesis is chromatin remodelling, to bring about long term changes in gene expression. I have therefore further analysed the function of NRSF, a well-

established coordinator of chromatin remodelling. The findings that NRSF isoforms can regulate pro-convulsant neuropeptide gene expression, and are potential targets for ACD action, strongly suggests that NRSF is of significance in epileptogenesis. This theory was further strengthen in chapter 7, whereby the modulation of NRSF isoforms, mirroring, in part that which occurs in response to KA-induced SSSE (Spencer *et al*., 2006), induced widespread changes in gene expression, modulating a host of neuronal genes with known association with epilepsy. Whilst these findings were obtained from a neuroblastoma cell line, and not a true SE model, they do provide support for the theory that NRSF is of particular importance in governing gene expression changes in response to an initial seizure, which could coordinate long term changes in gene expression profiles. It would therefore be of significant interest to take this work forward and experiment in a more appropriate *in vivo* model.

The use of a microarray revealed that a host of genes were modulated following over-expression of both full-length NRSF and a truncated version lacking the C-terminal repressor domain, analogous to sNRSF. This included ion channels (calcium channels, potassium channels, sodium channels and chloride channels), GABA transporters and receptors, glutamate receptors, drug metabolising enzymes, neuropeptide receptors and neurotrophic factors. RT-PCR was used to validate the microarray findings. This dataset indicates that the modulation of the NRSF isoforms has a dramatic effect on neuronal gene expression, altering the expression of a vast number of genes, which would have a significant impact upon neuronal phenotype. The change in ion channel expression alone may have a severe impact upon the electrical potential of the neurons, affecting their ability to fire. This may provide an explanation (or future experimentation) as to why

neurons become more susceptible to future seizures or abnormal firing, following an initial insult. Perhaps most importantly, are the findings that the different NRSF isoforms induce different gene expression patterns, in a temporal manner. Our group has previously proposed that the truncated isoform is important in seizure, and have postulated that it is the ratio between the two isoforms that is key in SSSE (Spencer *et al*., 2006). These findings support such a notion, and highlight the requirement to further explore the impact of this truncated isoform on the NRSF regulatory system. It is perhaps important to note that I have yet to explore the impact of USF over-expression in such a manner, and this is perhaps another importance experiment to help uncover the possible mechanisms at work during epileptogenesis. Furthermore, it is important to stress that these experiments were carried out in human neuroblastoma cell lines, and whilst they may provide the basis of new theories regarding the role of these TFs in epileptogenesis, they are in no way conclusive, and should be explored further in *in vivo* seizure models.

9.4 Cocaine modulates NRSF & USF

Due to an increasing appreciation of the role of the dopaminergic system in seizure, I sought to explore the impact of cocaine, a known modulator of the dopaminergic system, upon the NRSF and USF regulatory systems. Cocaine treatment was found to repress the expression of NRSF and, to a less significant extent, sNRSF, which corresponded, in part to an elevation in CART mRNA expression. This elevation in CART expression is thought to occur through the direct reduction of NRSF binding to the CART NRSE, leading to de-repression, however the preliminary ChIP assays failed to provide sufficient evidence to support this claim. Cocaine was found to also impair NRSF binding to the TAC1, NKB and BDNF NRSEs. This impairment however did not

affect the endogenous expression of NKB, presumably due to the repression of both USF1 and USF2 mRNA expression, supporting the earlier claims in chapter 3, that the USF TFs repress NKB expression. These findings reveal that cocaine modulate both the NRSF and USF regulatory systems, and perhaps controversially suggests that cocaine may elicit anti-convulsant properties, as its affect upon these TFs is in keeping to that elicited by the three ACDs tested.

9.5 Relevance to epilepsy

This thesis set out to explore the regulatory systems which may be relevant to epilepsy, and has provided support for the theory that both NRSF and USF TF families play a fundamental role in governing gene expression changes associated with epileptogenesis. Both TFs are shown here to be of importance in the regulation of two pro-convulsant neuropeptide encoding genes, TAC3 and TAC1, with NRSF and USF shown to cooperatively regulate the latter, in the cell line model employed here. I have proposed that these TFs fit the model of epileptogenesis, as both TF families are induced following an initial insult (such as KA-induced seizure), and are capable of orchestrating changes in gene expression of a plethora of neuronal genes. After a prolonged latent period, such changes in gene expression patterns, could have a dramatic impact upon neuronal phenotype, making the neurons more prone to future abnormal firing and hence manifesting in seizures.

In support of the potential importance of these distinct TFs in epileptogenesis, ACD treatment is shown to modulate the expression of these TFs in neuroblastoma cells. These findings represent a novel mechanism of action for the three ACDs tested here, and highlight the fact that these drugs work through multiple targets. Furthermore, the

importance of NRSF isoforms in governing changes in gene expression, with relevance to epilepsy, was highlighted through the use of microarray technology. Elevated expression of NRSF variants, a known response to SSSE (Spencer *et al*., 2006) is shown to modulate the expression of a multitude of genes with known associations with epilepsy. This suggests that NRSF may have an important role in governing gene expression responses in relation to epilepsy and epileptogenesis, however this needs to be explored further in an *in vivo* SE model. Finally, I have shown that cocaine, which has been linked with inducing seizures (Lowenstein *et al*., 1987; Hanson *et al*., 1999; Lason., 2001), also modulates both the NRSF and USF regulatory systems, further strengthening the theory that these TFs are of particular importance in orchestrating molecular changes in response to an initial insult or seizure.

Reference List

Abou-Khalil, B., Andermann, E., Andermann, F., Olivier, A. and Quesney, L. F., 1993. Temporal lobe epilepsy after prolonged febrile convulsions: excellent outcome after surgical treatment. *Epilepsia*. **34**, 878-883.

Abramovitz, L., Shapira, T., Ben-Dror, I., Dror, V., Granot, L., Rousso, T., Landoy, E., Blau, L., Thiel, G. and Vardimon, L., 2008. Dual role of NRSF/REST in activation and repression of the glucocorticoid response. *J Biol Chem*. **283**, 110-119.

Abuhatzira, L., Makedonski, K., Kaufman, Y., Razin, A. and Shemer, R., 2007. MeCP2 deficiency in the brain decreases BDNF levels by REST/CoREST-mediated repression and increases TRKB production. *Epigenetics*. **2**, 214-222.

Adams, C. C. and Workman, J. L., 1995. Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol Cell Biol*. **15**, 1405-1421.

Agalioti, T., Chen, G. and Thanos, D., 2002. Deciphering the transcriptional histone acetylation code for a human gene. *Cell*. **111**, 381-392.

Aksan, I. and Goding, C. R., 1998. Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-box elements in vitro and in vivo. *Mol Cell Biol*. **18**, 6930-6938.

Ambrosio, A. F., Soares-Da-Silva, P., Carvalho, C. M. and Carvalho, A. P., 2002. Mechanisms of action of carbamazepine and its derivatives, oxcarbazepine, BIA 2-093, and BIA 2-024. *Neurochem Res*. **27**, 121-130.

Andres, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., Dallman, J., Ballas, N. and Mandel, G., 1999. CoREST: a functional corepressor required for regulation of neural-specific gene expression. *Proc Natl Acad Sci U S A*. **96**, 9873-9878.

Andrews, G. K., Lee, D. K., Ravindra, R., Lichtlen, P., Sirito, M., Sawadogo, M. and Schaffner, W., 2001. The transcription factors MTF-1 and USF1 cooperate to regulate mouse metallothionein-I expression in response to the essential metal zinc in visceral endoderm cells during early development. *Embo J*. **20**, 1114-1122.

Aperlo, C., Boulukos, K. E. and Pognonec, P., 1996. The basic region/helix-loop-helix/leucine repeat transcription factor USF interferes with Ras transformation. *Eur J Biochem*. **241**, 249-253.

Arents, G., Burlingame, R. W., Wang, B. C., Love, W. E. and Moudrianakis, E. N., 1991. The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix. *Proc Natl Acad Sci U S A*. **88**, 10148-10152.

Arora, D. K., Cosgrave, A. S., Howard, M. R., Bubb, V., Quinn, J. P. and Thippeswamy, T., 2007. Evidence of postnatal neurogenesis in dorsal root ganglion: role of nitric oxide and neuronal restrictive

silencer transcription factor. *J Mol Neurosci*. **32**, 97-107.

Auvin, S., 2008. Treatment of juvenile myoclonic epilepsy. *CNS Neurosci Ther*. **14**, 227-233.

Bahn, S., Mimmack, M., Ryan, M., Caldwell, M. A., Jauniaux, E., Starkey, M., Svendsen, C. N. and Emson, P., 2002. Neuronal target genes of the neuron-restrictive silencer factor in neurospheres derived from fetuses with Down's syndrome: a gene expression study. *Lancet*. **359**, 310-315.

Ballas, N., Grunseich, C., Lu, D. D., Speh, J. C. and Mandel, G., 2005. REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell*. **121**, 645-657.

Ballas, N. and Mandel, G., 2005. The many faces of REST oversee epigenetic programming of neuronal genes. *Curr Opin Neurobiol*. **15**, 500-506.

Bannister, A. J. and Kouzarides, T., 2005. Reversing histone methylation. *Nature*. **436**, 1103-1106.

Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C. and Kouzarides, T., 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*. **410**, 120-124.

Baraban, S. C., 2004. Neuropeptide Y and epilepsy: recent progress, prospects and controversies. *Neuropeptides*. **38**, 261-265.

Barker, R., 1991. Substance P and neurodegenerative disorders. A speculative review. *Neuropeptides*. **20**, 73-78.

Bassuk, A. G., Wallace, R. H., Buhr, A., Buller, A. R., Afawi, Z., Shimojo, M., Miyata, S., Chen, S., Gonzalez-Alegre, P., Griesbach, H. L., Wu, S., Nashelsky, M., Vladar, E. K., Antic, D., Ferguson, P. J., Cirak, S., Voit, T., Scott, M. P., Axelrod, J. D., Gurnett, C., Daoud, A. S., Kivity, S., Neufeld, M. Y., Mazarib, A., Straussberg, R., Walid, S., Korczyn, A. D., Slusarski, D. C., Berkovic, S. F. and El-Shanti, H. I., 2008. A homozygous mutation in human PRICKLE1 causes an autosomal-recessive progressive myoclonus epilepsy-ataxia syndrome. *Am J Hum Genet*. **83**, 572-581.

Battaglioli, E., Andres, M. E., Rose, D. W., Chenoweth, J. G., Rosenfeld, M. G., Anderson, M. E. and Mandel, G., 2002. REST repression of neuronal genes requires components of the hSWI.SNF complex. *J Biol Chem*. **277**, 41038-41045.

Becker, A. J., Wiestler, O. D. and Blumcke, I., 2002. Functional genomics in experimental and human temporal lobe epilepsy: powerful new tools to identify molecular disease mechanisms of hippocampal damage. *Prog Brain Res*. **135**, 161-173.

Bell, G. S. and Sander, J. W., 2001. The epidemiology of epilepsy: the size of the problem. *Seizure*. **10**, 306-314; quiz 315-306.

Belmont, A. S. and Bruce, K., 1994. Visualization of G1 chromosomes: a folded, twisted, supercoiled chromonema model of interphase chromatid structure. *J Cell Biol*. **127**, 287-302.

Belyaev, N. D., Wood, I. C., Bruce, A. W., Street, M., Trinh, J. B. and Buckley, N. J., 2004. Distinct RE-1 silencing transcription factor-containing complexes interact with different target genes. *J Biol Chem*. **279**,

556-561.

Bengzon, J., Kokaia, Z., Ernfors, P., Kokaia, M., Leanza, G., Nilsson, O. G., Persson, H. and Lindvall, O., 1993. Regulation of neurotrophin and trkA, trkB and trkC tyrosine kinase receptor messenger RNA expression in kindling. *Neuroscience*. **53**, 433-446.

Berkovic, S. F., Izzillo, P., McMahon, J. M., Harkin, L. A., McIntosh, A. M., Phillips, H. A., Briellmann, R. S., Wallace, R. H., Mazarib, A., Neufeld, M. Y., Korczyn, A. D., Scheffer, I. E. and Mulley, J. C., 2004. LGI1 mutations in temporal lobe epilepsies. *Neurology*. **62**, 1115-1119.

Bessis, A., Champtiaux, N., Chatelin, L. and Changeux, J. P., 1997. The neuron-restrictive silencer element: a dual enhancer/silencer crucial for patterned expression of a nicotinic receptor gene in the brain. *Proc Natl Acad Sci U S A*. **94**, 5906-5911.

Biedler, J. L., Roffler-Tarlov, S., Schachner, M. and Freedman, L. S., 1978. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res*. **38**, 3751-3757.

Bilkey, D. K. and Clearwater, J. M., 2005. The dynamic nature of spatial encoding in the hippocampus. *Behav Neurosci*. **119**, 1533-1545.

Bingham, A. J., Ooi, L. and Wood, I. C., 2006. Multiple chromatin modifications important for gene expression changes in cardiac hypertrophy. *Biochem Soc Trans*. **34**, 1138-1140.

Blackstad, T. W., Brink, K., Hem, J., & Jeune, B. , 1970. Distribution of hippocampal mossy fibers in the rat. An experimental study with silver impregnation methods. *J Comp Neurol*. **138**, 433-449.

Bliss, T. V. and Collingridge, G. L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*. **361**, 31-39.

Blumenfeld, H., Lampert, A., Klein, J. P., Mission, J., Chen, M. C., Rivera, M., Dib-Hajj, S., Brennan, A. R., Hains, B. C. and Waxman, S. G., 2009. Role of hippocampal sodium channel Nav1.6 in kindling epileptogenesis. *Epilepsia*. **50**, 44-55.

Brady, K. T., Sonne, S. C., Malcolm, R. J., Randall, C. L., Dansky, B. S., Simpson, K., Roberts, J. S. and Brondino, M., 2002. Carbamazepine in the treatment of cocaine dependence: subtyping by affective disorder. *Exp Clin Psychopharmacol*. **10**, 276-285.

Brene, S., Lindefors, N., Ballarin, M. and Persson, H., 1992. Kainic acid-mediated increase of preprotachykinin-A messenger RNA expression in the rat hippocampus and a region-selective attenuation by dexamethasone. *Neuroscience*. **50**, 611-618.

Briellmann, R. S., Berkovic, S. F., Syngeniotis, A., King, M. A. and Jackson, G. D., 2002. Seizure-associated hippocampal volume loss: a longitudinal magnetic resonance study of temporal lobe epilepsy. *Ann Neurol*. **51**, 641-644.

Brodie, M. J., Richens, A. and Yuen, A. W., 1995. Double-blind comparison of lamotrigine and carbamazepine in newly diagnosed epilepsy. UK Lamotrigine/Carbamazepine Monotherapy Trial Group. *Lancet*. **345**, 476-479.

Bruce, A. W., Donaldson, I. J., Wood, I. C., Yerbury, S. A., Sadowski, M. I., Chapman, M., Gottgens, B. and Buckley, N. J., 2004. Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc Natl Acad Sci U S A*. **101**, 10458-10463.

Bruce, A. W., Krejci, A., Ooi, L., Deuchars, J., Wood, I. C., Dolezal, V. and Buckley, N. J., 2006. The transcriptional repressor REST is a critical regulator of the neurosecretory phenotype. *J Neurochem*. **98**, 1828-1840.

Bruno, M., Flaus, A., Stockdale, C., Rencurel, C., Ferreira, H. and Owen-Hughes, T., 2003. Histone H2A/H2B dimer exchange by ATP-dependent chromatin remodeling activities. *Mol Cell*. **12**, 1599-1606.

Buell, G., Schulz, M. F., Arkinstall, S. J., Maury, K., Missotten, M., Adami, N., Talabot, F. and Kawashima, E., 1992. Molecular characterisation, expression and localisation of human neurokinin-3 receptor. *FEBS Lett*. **299**, 90-95.

Calin-Jageman, I. E., Wang, J. and Bannon, M. J., 2006. Regulation of the preprotachykinin-I gene promoter through a protein kinase A-dependent, cyclic AMP response element-binding protein-independent mechanism. *J Neurochem*. **97**, 255-264.

Campbell, J., Nickel, E. J., Penick, E. C., Wallace, D., Gabrielli, W. F., Rowe, C., Liskow, B., Powell, B. J. and Thomas, H. M., 2003. Comparison of desipramine or carbamazepine to placebo for crack cocaine-dependent patients. *Am J Addict*. **12**, 122-136.

Cannarsa, R., Landuzzi, D., Cavina, C., Candeletti, S. and Romualdi, P., 2008. Kainic acid down-regulates NOP receptor density and gene expression in human neuroblastoma SH-SY5Y cells. *J Mol Neurosci*. **35**, 171-177.

Canzonetta, C., Mulligan, C., Deutsch, S., Ruf, S., O'Doherty, A., Lyle, R., Borel, C., Lin-Marq, N., Delom, F., Groet, J., Schnappauf, F., De Vita, S., Averill, S., Priestley, J. V., Martin, J. E., Shipley, J., Denyer, G., Epstein, C. J., Fillat, C., Estivill, X., Tybulewicz, V. L., Fisher, E. M., Antonarakis, S. E. and Nizetic, D., 2008. DYRK1A-dosage imbalance perturbs NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate in Down syndrome. *Am J Hum Genet*. **83**, 388-400.

Cao, Y. Q., Mantyh, P. W., Carlson, E. J., Gillespie, A. M., Epstein, C. J. and Basbaum, A. I., 1998. Primary afferent tachykinins are required to experience moderate to intense pain. *Nature*. **392**, 390-394.

Carter, M. S. and Krause, J. E., 1990. Structure, expression, and some regulatory mechanisms of the rat preprotachykinin gene encoding substance P, neurokinin A, neuropeptide K, and neuropeptide gamma. *J Neurosci*. **10**, 2203-2214.

Carthew, R. W., Chodosh, L. A. and Sharp, P. A., 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. *Cell*. **43**, 439-448.

Cavalleri, G. L., Weale, M. E., Shianna, K. V., Singh, R., Lynch, J. M., Grinton, B., Szoeke, C., Murphy, K., Kinirons, P., O'Rourke, D., Ge, D., Depondt, C., Claeys, K. G., Pandolfo, M., Gumbs, C., Walley, N., McNamara, J., Mulley, J. C., Linney, K. N., Sheffield, L. J., Radtke, R. A., Tate, S. K., Chissoe, S. L., Gibson, R. A., Hosford, D., Stanton, A., Graves, T. D., Hanna, M. G., Eriksson, K., Kantanen, A. M., Kalviainen, R., O'Brien, T. J., Sander, J. W., Duncan, J. S., Scheffer, I. E., Berkovic, S. F., Wood, N. W.,

Doherty, C. P., Delanty, N., Sisodiya, S. M. and Goldstein, D. B., 2007. Multicentre search for genetic susceptibility loci in sporadic epilepsy syndrome and seizure types: a case-control study. *Lancet Neurol*. **6**, 970-980.

Chang, J. T., Yang, H. T., Wang, T. C. and Cheng, A. J., 2005. Upstream stimulatory factor (USF) as a transcriptional suppressor of human telomerase reverse transcriptase (hTERT) in oral cancer cells. *Mol Carcinog*. **44**, 183-192.

Chang, Y. C., Rapoport, S. I. and Rao, J. S., 2009. Chronic administration of mood stabilizers upregulates BDNF and bcl-2 expression levels in rat frontal cortex. *Neurochem Res*. **34**, 536-541.

Chawla, M. K., Gutierrez, G. M., Young, W. S., 3rd, McMullen, N. T. and Rance, N. E., 1997. Localization of neurons expressing substance P and neurokinin B gene transcripts in the human hypothalamus and basal forebrain. *J Comp Neurol*. **384**, 429-442.

Chen, J. W. and Wasterlain, C. G., 2006. Status epilepticus: pathophysiology and management in adults. *Lancet Neurol*. **5**, 246-256.

Chen, L., Shen, Y. H., Wang, X., Wang, J., Gan, Y., Chen, N., Wang, J., LeMaire, S. A., Coselli, J. S. and Wang, X. L., 2006. Human prolyl-4-hydroxylase alpha(I) transcription is mediated by upstream stimulatory factors. *J Biol Chem*. **281**, 10849-10855.

Chen, L. W., Wang, Y. Q., Bian, G. L., Wei, L. C. and Yung, K. L., 2008. Neurokinin-3 peptide instead of neurokinin-1 synergistically exacerbates kainic acid-inducing degeneration of neurons in the substantia nigra of mice. *J Neurochem*. **105**, 203-216.

Chen, L. W., Yung, K. K. and Chan, Y. S., 2004. Neurokinin peptides and neurokinin receptors as potential therapeutic intervention targets of basal ganglia in the prevention and treatment of Parkinson's disease. *Curr Drug Targets*. **5**, 197-206.

Chen, Z. F., Paquette, A. J. and Anderson, D. J., 1998. NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat Genet*. **20**, 136-142.

Cheong, A., Bingham, A. J., Li, J., Kumar, B., Sukumar, P., Munsch, C., Buckley, N. J., Neylon, C. B., Porter, K. E., Beech, D. J. and Wood, I. C., 2005. Downregulated REST transcription factor is a switch enabling critical potassium channel expression and cell proliferation. *Mol Cell*. **20**, 45-52.

Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altshuller, Y. M., Frohman, M. A., Kraner, S. D. and Mandel, G., 1995. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell*. **80**, 949-957.

Collins, R. C., Tearse, R. G., & Lothman, E. W, 1983. Functional anatomy of limbic seizures: focal discharges from medial entorhinal cortex in rat. *Brain Res* **280**, 25-40.

Corcoran, K. E., Malhotra, A., Molina, C. A. and Rameshwar, P., 2008. Stromal-derived factor-1alpha induces a non-canonical pathway to activate the endocrine-linked Tac1 gene in non-tumorigenic breast cells. *J Mol Endocrinol*. **40**, 113-123.

Corre, S. and Galibert, M. D., 2005. Upstream stimulating factors: highly versatile stress-responsive

transcription factors. *Pigment Cell Res*. **18**, 337-348.

Cortez, M. A., Wu, Y., Gibson, K. M. and Snead, O. C., 3rd, 2004. Absence seizures in succinic semialdehyde dehydrogenase deficient mice: a model of juvenile absence epilepsy. *Pharmacol Biochem Behav*. **79**, 547-553.

Coulson, J. M., Ahmed, S. I., Quinn, J. P. and Woll, P. J., 2003. Detection of small cell lung cancer by RT-PCR for neuropeptides, neuropeptide receptors, or a splice variant of the neuron restrictive silencer factor. *Methods Mol Med*. **75**, 335-352.

Coulson, J. M., Edgson, J. L., Marshall-Jones, Z. V., Mulgrew, R., Quinn, J. P. and Woll, P. J., 2003. Upstream stimulatory factor activates the vasopressin promoter via multiple motifs, including a non-canonical E-box. *Biochem J*. **369**, 549-561.

Coulson, J. M., Edgson, J. L., Woll, P. J. and Quinn, J. P., 2000. A splice variant of the neuron-restrictive silencer factor repressor is expressed in small cell lung cancer: a potential role in derepression of neuroendocrine genes and a useful clinical marker. *Cancer Res*. **60**, 1840-1844.

Coulson, J. M., Fiskerstrand, C. E., Woll, P. J. and Quinn, J. P., 1999. Arginine vasopressin promoter regulation is mediated by a neuron-restrictive silencer element in small cell lung cancer. *Cancer Res*. **59**, 5123-5127.

Croll, S. D., Chesnutt, C. R., Greene, N. A., Lindsay, R. M. and Wiegand, S. J., 1999. Peptide immunoreactivity in aged rat cortex and hippocampus as a function of memory and BDNF infusion. *Pharmacol Biochem Behav*. **64**, 625-635.

Crunelli, V. and Leresche, N., 2002. Childhood absence epilepsy: genes, channels, neurons and networks. *Nat Rev Neurosci*. **3**, 371-382.

Crusselle-Davis, V. J., Vieira, K. F., Zhou, Z., Anantharaman, A. and Bungert, J., 2006. Antagonistic regulation of beta-globin gene expression by helix-loop-helix proteins USF and TFII-I. *Mol Cell Biol*. **26**, 6832-6843.

Cunningham, M. O., Dhillon, A., Wood, S. J. and Jones, R. S., 2000. Reciprocal modulation of glutamate and GABA release may underlie the anticonvulsant effect of phenytoin. *Neuroscience*. **95**, 343-351.

Cunningham, M. O. and Jones, R. S., 2000. The anticonvulsant, lamotrigine decreases spontaneous glutamate release but increases spontaneous GABA release in the rat entorhinal cortex in vitro. *Neuropharmacology*. **39**, 2139-2146.

Davidson, C., Lee, T. H. and Ellinwood, E. H., 2004. The NK(1) receptor antagonist WIN51708 reduces sensitization after chronic cocaine. *Eur J Pharmacol*. **499**, 355-356.

de Boer, H. M., Mula, M. and Sander, J. W., 2008. The global burden and stigma of epilepsy. *Epilepsy Behav*. **12**, 540-546.

de Souza Silva, M. A., Mello, E. L., Jr., Muller, C. P., Jocham, G., Maior, R. S., Huston, J. P., Tomaz, C. and Barros, M., 2006. Interaction of the tachykinin NK3 receptor agonist senktide with behavioral effects of cocaine in marmosets (Callithrix penicillata). *Peptides*. **27**, 2214-2223.

deGraffenried, L. A., Hopp, T. A., Valente, A. J., Clark, R. A. and Fuqua, S. A., 2004. Regulation of the estrogen receptor alpha minimal promoter by Sp1, USF-1 and ERalpha. *Breast Cancer Res Treat*. **85**, 111-120.

Dellovade, T. L. and Merchenthaler, I., 2004. Estrogen regulation of neurokinin B gene expression in the mouse arcuate nucleus is mediated by estrogen receptor alpha. *Endocrinology*. **145**, 736-742.

Ding, N., Tomomori-Sato, C., Sato, S., Conaway, R. C., Conaway, J. W. and Boyer, T. G., 2009. MED19 and MED26 are synergistic functional targets of the RE1 silencing transcription factor in epigenetic silencing of neuronal gene expression. *J Biol Chem*. **284**, 2648-2656.

Ding, N., Zhou, H., Esteve, P. O., Chin, H. G., Kim, S., Xu, X., Joseph, S. M., Friez, M. J., Schwartz, C. E., Pradhan, S. and Boyer, T. G., 2008. Mediator links epigenetic silencing of neuronal gene expression with x-linked mental retardation. *Mol Cell*. **31**, 347-359.

Dore, S., Kar, S. and Quirion, R., 1997. Rediscovering an old friend, IGF-I: potential use in the treatment of neurodegenerative diseases. *Trends Neurosci*. **20**, 326-331.

Douglass, J., McKinzie, A. A. and Couceyro, P., 1995. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J Neurosci*. **15**, 2471-2481.

Drews, V. L., Shi, K., de Haan, G. and Meisler, M. H., 2007. Identification of evolutionarily conserved, functional noncoding elements in the promoter region of the sodium channel gene SCN8A. *Mamm Genome*. **18**, 723-731.

Duggan, A. W., Hendry, I. A., Morton, C. R., Hutchison, W. D. and Zhao, Z. Q., 1988. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. *Brain Res*. **451**, 261-273.

Duggan, A. W., Morton, C. R., Zhao, Z. Q. and Hendry, I. A., 1987. Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. *Brain Res*. **403**, 345-349.

Ehrhard, P. B., Ganter, U., Schmutz, B., Bauer, J. and Otten, U., 1993. Expression of low-affinity NGF receptor and trkB mRNA in human SH-SY5Y neuroblastoma cells. *FEBS Lett*. **330**, 287-292.

El-Badry, O. M., Romanus, J. A., Helman, L. J., Cooper, M. J., Rechler, M. M. and Israel, M. A., 1989. Autonomous growth of a human neuroblastoma cell line is mediated by insulin-like growth factor II. *J Clin Invest*. **84**, 829-839.

Elliott, P., 1990. Action of antiepileptic and anaesthetic drugs on Na- and Ca-spikes in mammalian non-myelinated axons. *Eur J Pharmacol*. **175**, 155-163.

Elliott, R. C., Khademi, S., Pleasure, S. J., Parent, J. M. and Lowenstein, D. H., 2001. Differential regulation of basic helix-loop-helix mRNAs in the dentate gyrus following status epilepticus. *Neuroscience*. **106**, 79-88.

Esclapez, M., Hirsch, J. C., Khazipov, R., Ben-Ari, Y. and Bernard, C., 1997. Operative GABAergic inhibition in hippocampal CA1 pyramidal neurons in experimental epilepsy. *Proc Natl Acad Sci U S A*. **94**, 12151-12156.

Ettinger, A. B. and Argoff, C. E., 2007. Use of antiepileptic drugs for nonepileptic conditions: psychiatric disorders and chronic pain. *Neurotherapeutics*. **4**, 75-83.

Everett, K., Chioza, B., Aicardi, J., Aschauer, H., Brouwer, O., Callenbach, P., Covanis, A., Dooley, J., Dulac, O., Durner, M., Eeg-Olofsson, O., Feucht, M., Friis, M., Guerrini, R., Heils, A., Kjeldsen, M., Nabbout, R., Sander, T., Wirrell, E., McKeigue, P., Robinson, R., Taske, N. and Gardiner, M., 2007. Linkage and mutational analysis of CLCN2 in childhood absence epilepsy. *Epilepsy Res*. **75**, 145-153.

Feen, E. S., Bershad, E. M. and Suarez, J. I., 2008. Status epilepticus. *South Med J*. **101**, 400-406.

Filip, M., Faron-Gorecka, A., Kusmider, M., Golda, A., Frankowska, M. and Dziedzicka-Wasylewska, M., 2006. Alterations in BDNF and trkB mRNAs following acute or sensitizing cocaine treatments and withdrawal. *Brain Res*. **1071**, 218-225.

Fisher, F., Crouch, D. H., Jayaraman, P. S., Clark, W., Gillespie, D. A. and Goding, C. R., 1993. Transcription activation by Myc and Max: flanking sequences target activation to a subset of CACGTG motifs in vivo. *Embo J*. **12**, 5075-5082.

Fisher, J. L., 2009. The anti-convulsant stiripentol acts directly on the GABA(A) receptor as a positive allosteric modulator. *Neuropharmacology*. **56**, 190-197.

Fisher, R. S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P. and Engel, J., Jr., 2005. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia*. **46**, 470-472.

Fiskerstrand, C. and Quinn, J. P., 1996. The molecular biology of preprotachykinin-A gene expression. *Neuropeptides*. **30**, 602-610.

Fiskerstrand, C. E., Lovejoy, E., Gerrard, L. and Quinn, J. P., 1999. An intronic domain within the rat preprotachykinin-A gene containing a CCCT repetitive motif acts as an enhancer in differentiating embryonic stem cells. *Neurosci Lett*. **263**, 141-144.

Fiskerstrand, C. E., Newey, P., McGregor, G. P., Gerrard, L., Millan, F. and Quinn, J. P., 2000. A role for Octamer binding protein motifs in the regulation of the proximal preprotachykinin-A promoter. *Neuropeptides*. **34**, 348-354.

Floor, E., Grad, O. and Leeman, S. E., 1982. Synaptic vesicles containing substance P purified by chromatography on controlled pore glass. *Neuroscience*. **7**, 1647-1655.

Formisano, L., Noh, K. M., Miyawaki, T., Mashiko, T., Bennett, M. V. and Zukin, R. S., 2007. Ischemic insults promote epigenetic reprogramming of mu opioid receptor expression in hippocampal neurons. *Proc Natl Acad Sci U S A*. **104**, 4170-4175.

Frenkel, S., Kay, G., Nechushtan, H. and Razin, E., 1998. Nuclear translocation of upstream stimulating factor 2 (USF2) in activated mast cells: a possible role in their survival. *J Immunol*. **161**, 2881-2887.

Freund, T. F., Hajos, N., Acsady, L., Gorcs, T. J. and Katona, I., 1997. Mossy cells of the rat dentate gyrus are immunoreactive for calcitonin gene-related peptide (CGRP). *Eur J Neurosci*. **9**, 1815-1830.

Friederich, P., Benzenberg, D. and Urban, B. W., 2002. Bupivacaine inhibits human neuronal Kv3 channels in SH-SY5Y human neuroblastoma cells. *Br J Anaesth*. **88**, 864-866.

Fujii, G., Nakamura, Y., Tsukamoto, D., Ito, M., Shiba, T. and Takamatsu, N., 2006. CpG methylation at the USF-binding site is important for the liver-specific transcription of the chipmunk HP-27 gene. *Biochem J*. **395**, 203-209.

Fujimi, T. J. and Aruga, J., 2008. Upstream stimulatory factors, USF1 and USF2 are differentially expressed during Xenopus embryonic development. *Gene Expr Patterns*. **8**, 376-381.

Fukuchi, M., Nii, T., Ishimaru, N., Minamino, A., Hara, D., Takasaki, I., Tabuchi, A. and Tsuda, M., 2009. Valproic acid induces up- or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through its epigenetic actions. *Neurosci Res*. **65**, 35-43.

Fuller, G. N., Su, X., Price, R. E., Cohen, Z. R., Lang, F. F., Sawaya, R. and Majumder, S., 2005. Many human medulloblastoma tumors overexpress repressor element-1 silencing transcription (REST)/neuron-restrictive silencer factor, which can be functionally countered by REST-VP16. *Mol Cancer Ther*. **4**, 343-349.

Furuta, T. and Kaneko, T., 2006. Third pathway in the cortico-basal ganglia loop: Neurokinin B-producing striatal neurons modulate cortical activity via striato-innominato-cortical projection. *Neurosci Res*. **54**, 1-10.

Gale, K., 1992. GABA and epilepsy: basic concepts from preclinical research. *Epilepsia*. **33 Suppl 5**, S3-12.

Galibert, M. D., Carreira, S. and Goding, C. R., 2001. The Usf-1 transcription factor is a novel target for the stress-responsive p38 kinase and mediates UV-induced Tyrosinase expression. *Embo J*. **20**, 5022-5031.

Gallanti, A., Tonelli, A., Cardin, V., Bussone, G., Bresolin, N. and Bassi, M. T., 2008. A novel de novo nonsense mutation in ATP1A2 associated with sporadic hemiplegic migraine and epileptic seizures. *J Neurol Sci*. **273**, 123-126.

Gambardella, A., Manna, I., Labate, A., Chifari, R., La Russa, A., Serra, P., Cittadella, R., Bonavita, S., Andreoli, V., LePiane, E., Sasanelli, F., Di Costanzo, A., Zappia, M., Tedeschi, G., Aguglia, U. and Quattrone, A., 2003. GABA(B) receptor 1 polymorphism (G1465A) is associated with temporal lobe epilepsy. *Neurology*. **60**, 560-563.

Gantois, I., Fang, K., Jiang, L., Babovic, D., Lawrence, A. J., Ferreri, V., Teper, Y., Jupp, B., Ziebell, J., Morganti-Kossmann, C. M., O'Brien, T. J., Nally, R., Schutz, G., Waddington, J., Egan, G. F. and Drago, J., 2007. Ablation of D1 dopamine receptor-expressing cells generates mice with seizures, dystonia, hyperactivity, and impaired oral behavior. *Proc Natl Acad Sci U S A*. **104**, 4182-4187.

Garriga-Canut, M., Schoenike, B., Qazi, R., Bergendahl, K., Daley, T. J., Pfender, R. M., Morrison, J. F., Ockuly, J., Stafstrom, C., Sutula, T. and Roopra, A., 2006. 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat Neurosci*. **9**, 1382-1387.

Gastaldi, M., Robaglia-Schlupp, A., Massacrier, A., Planells, R. and Cau, P., 1998. mRNA coding for voltage-gated sodium channel beta2 subunit in rat central nervous system: cellular distribution and changes following kainate-induced seizures. *Neurosci Lett*. **249**, 53-56.

Gerard, N. P., Eddy, R. L., Jr., Shows, T. B. and Gerard, C., 1990. The human neurokinin A (substance K) receptor. Molecular cloning of the gene, chromosome localization, and isolation of cDNA from tracheal and gastric tissues. *J Biol Chem*. **265**, 20455-20462.

Gerard, N. P., Garraway, L. A., Eddy, R. L., Jr., Shows, T. B., Iijima, H., Paquet, J. L. and Gerard, C., 1991. Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones. *Biochemistry*. **30**, 10640-10646.

Gerrard, L., Howard, M., Paterson, T., Thippeswamy, T., Quinn, J. P. and Haddley, K., 2005. A proximal E-box modulates NGF effects on rat PPT-A promoter activity in cultured dorsal root ganglia neurones. *Neuropeptides*. **39**, 475-483.

Greene, L. A. and Tischler, A. S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A*. **73**, 2424-2428.

Glisovic, S., Glavan, G., Saghafi, M. M. and Zivin, M., 2007. Upregulation of synaptotagmin IV protein in kainate-induced seizures. *Neuroreport*. **18**, 831-835.

Gong, H. C., Hang, J., Kohler, W., Li, L. and Su, T. Z., 2001. Tissue-specific expression and gabapentin-binding properties of calcium channel alpha2delta subunit subtypes. *J Membr Biol*. **184**, 35-43.
Gonzalez-Martinez, J. A., Bingaman, W. E., Toms, S. A. and Najm, I. M., 2007. Neurogenesis in the postnatal human epileptic brain. *J Neurosurg*. **107**, 628-635.

Goueli, B. S. and Janknecht, R., 2003. Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. *Oncogene*. **22**, 8042-8047.

Greco, S. J., Smirnov, S. V., Murthy, R. G. and Rameshwar, P., 2007. Synergy between the RE-1 silencer of transcription and NFkappaB in the repression of the neurotransmitter gene TAC1 in human mesenchymal stem cells. *J Biol Chem*. **282**, 30039-30050.

Greenway, D. J., Street, M., Jeffries, A. and Buckley, N. J., 2007. RE1 Silencing transcription factor maintains a repressive chromatin environment in embryonic hippocampal neural stem cells. *Stem Cells*. **25**, 354-363.

Gregor, P. D., Sawadogo, M. and Roeder, R. G., 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. *Genes Dev*. **4**, 1730-1740.

Grimes, J. A., Nielsen, S. J., Battaglioli, E., Miska, E. A., Speh, J. C., Berry, D. L., Atouf, F., Holdener, B. C., Mandel, G. and Kouzarides, T., 2000. The co-repressor mSin3A is a functional component of the REST-CoREST repressor complex. *J Biol Chem*. **275**, 9461-9467.

Grimm, J. W., Lu, L., Hayashi, T., Hope, B. T., Su, T. P. and Shaham, Y., 2003. Time-dependent

increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. *J Neurosci*. **23**, 742-747.

Gruber, B., Greber, S., Rupp, E. and Sperk, G., 1994. Differential NPY mRNA expression in granule cells and interneurons of the rat dentate gyrus after kainic acid injection. *Hippocampus*. **4**, 474-482.

Gryder, D. S. and Rogawski, M. A., 2003. Selective antagonism of GluR5 kainate-receptor-mediated synaptic currents by topiramate in rat basolateral amygdala neurons. *J Neurosci*. **23**, 7069-7074.

Guardavaccaro, D., Frescas, D., Dorrello, N. V., Peschiaroli, A., Multani, A. S., Cardozo, T., Lasorella, A., Iavarone, A., Chang, S., Hernando, E. and Pagano, M., 2008. Control of chromosome stability by the beta-TrCP-REST-Mad2 axis. *Nature*. **452**, 365-369.

Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R. and Young, R. A., 2007. A chromatin landmark and transcription initiation at most promoters in human cells. *Cell*. **130**, 77-88.

Gupta, M., Polinsky, M., Senephansiri, H., Snead, O. C., Jansen, E. E., Jakobs, C. and Gibson, K. M., 2004. Seizure evolution and amino acid imbalances in murine succinate semialdehyde dehydrogenase (SSADH) deficiency. *Neurobiol Dis*. **16**, 556-562.

Gurpur, P. B., Liu, J., Burkin, D. J. and Kaufman, S. J., 2009. Valproic acid activates the PI3K/Akt/mTOR pathway in muscle and ameliorates pathology in a mouse model of Duchenne muscular dystrophy. *Am J Pathol*. **174**, 999-1008.

Gutierrez, R., Romo-Parra, H., Maqueda, J., Vivar, C., Ramirez, M., Morales, M. A. and Lamas, M., 2003. Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. *J Neurosci*. **23**, 5594-5598.

Gutierrez-Delicado, E. and Serratosa, J. M., 2004. Genetics of the epilepsies. *Curr Opin Neurol*. **17**, 147-153.

Halonen, T., Nissinen, J. and Pitkanen, A., 2001. Effect of lamotrigine treatment on status epilepticus-induced neuronal damage and memory impairment in rat. *Epilepsy Res*. **46**, 205-223.

Han, M. and Grunstein, M., 1988. Nucleosome loss activates yeast downstream promoters in vivo. *Cell*. **55**, 1137-1145.

Han, M., Kim, U. J., Kayne, P. and Grunstein, M., 1988. Depletion of histone H4 and nucleosomes activates the PHO5 gene in Saccharomyces cerevisiae. *Embo J*. **7**, 2221-2228.

Hanaya, R., Sasa, M., Kiura, Y., Serikawa, T. and Kurisu, K., 2002. Effects of vigabatrin on epileptiform abnormal discharges in hippocampal CA3 neurons of spontaneously epileptic rats (SER). *Epilepsy Res*. **50**, 223-231.

Hanson, G. R., Jensen, M., Johnson, M. and White, H. S., 1999. Distinct features of seizures induced by cocaine and amphetamine analogs. *Eur J Pharmacol*. **377**, 167-173.

Hara, D., Fukuchi, M., Miyashita, T., Tabuchi, A., Takasaki, I., Naruse, Y., Mori, N., Kondo, T. and Tsuda, M., 2009. Remote control of activity-dependent BDNF gene promoter-I transcription mediated by

REST/NRSF. *Biochem Biophys Res Commun*. **384**, 506-511.

Harrison, P. T., Dalziel, R. G., Ditchfield, N. A. and Quinn, J. P., 1999. Neuronal-specific and nerve growth factor-inducible expression directed by the preprotachykinin-A promoter delivered by an adeno-associated virus vector. *Neuroscience*. **94**, 997-1003.

Hatae, T., Kawano, H., Karpitskiy, V., Krause, J. E. and Masuko, S., 2001. Arginine-vasopressin neurons in the rat hypothalamus produce neurokinin B and co-express the tachykinin NK-3 receptor and angiotensin II type 1 receptor. *Arch Histol Cytol*. **64**, 37-44.

He, X. P., Kotloski, R., Nef, S., Luikart, B. W., Parada, L. F. and McNamara, J. O., 2004. Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. *Neuron*. **43**, 31-42.

Helbig, I., Matigian, N. A., Vadlamudi, L., Lawrence, K. M., Bayly, M. A., Bain, S. M., Diyagama, D., Scheffer, I. E., Mulley, J. C., Holloway, A. J., Dibbens, L. M., Berkovic, S. F. and Hayward, N. K., 2008. Gene expression analysis in absence epilepsy using a monozygotic twin design. *Epilepsia*. **49**, 1546-1554.

Henze, D. A., Urban, N. N. and Barrionuevo, G., 2000. The multifarious hippocampal mossy fiber pathway: a review. *Neuroscience*. **98**, 407-427.

Hermann, B. P., Hornbaker, K., Rice, D. A., Sawadogo, M. and Heckert, L. L., 2008. In vivo regulation of follicle-stimulating hormone receptor by the transcription factors upstream stimulatory factor 1 and upstream stimulatory factor 2 is cell specific. *Endocrinology*. **149**, 5297-5306.

Holstege, F. C., Fiedler, U. and Timmers, H. T., 1997. Three transitions in the RNA polymerase II transcription complex during initiation. *Embo J*. **16**, 7468-7480.

Howard, M. R., Millward-Sadler, S. J., Vasilliou, A. S., Salter, D. M. and Quinn, J. P., 2008. Mechanical stimulation induces preprotachykinin gene expression in osteoarthritic chondrocytes which is correlated with modulation of the transcription factor neuron restrictive silence factor. *Neuropeptides*. **42**, 681-686.

Huang, Y., Doherty, J. J. and Dingledine, R., 2002. Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. *J Neurosci*. **22**, 8422-8428.

Huang, Y., Myers, S. J. and Dingledine, R., 1999. Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. *Nat Neurosci*. **2**, 867-872.

Hubert, G. W. and Kuhar, M. J., 2005. Colocalization of CART with substance P but not enkephalin in the rat nucleus accumbens. *Brain Res*. **1050**, 8-14.

Huguenard, J. R., 1996. Low-threshold calcium currents in central nervous system neurons. *Annu Rev Physiol*. **58**, 329-348.

Hunsberger, J. G., Bennett, A. H., Selvanayagam, E., Duman, R. S. and Newton, S. S., 2005. Gene profiling the response to kainic acid induced seizures. *Brain Res Mol Brain Res*. **141**, 95-112.

Imbalzano, A. N., Kwon, H., Green, M. R. and Kingston, R. E., 1994. Facilitated binding of TATA-binding protein to nucleosomal DNA. *Nature*. **370**, 481-485.

Isackson, P. J., Huntsman, M. M., Murray, K. D. and Gall, C. M., 1991. BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. *Neuron*. **6**, 937-948.

Ismail, P. M., Lu, T. and Sawadogo, M., 1999. Loss of USF transcriptional activity in breast cancer cell lines. *Oncogene*. **18**, 5582-5591.

Jaiswal, A. S. and Narayan, S., 2001. Upstream stimulating factor-1 (USF1) and USF2 bind to and activate the promoter of the adenomatous polyposis coli (APC) tumor suppressor gene. *J Cell Biochem*. **81**, 262-277.

Jamsa, A., Backstrom, A., Gustafsson, E., Dehvari, N., Hiller, G., Cowburn, R. F. and Vasange, M., 2006. Glutamate treatment and p25 transfection increase Cdk5 mediated tau phosphorylation in SH-SY5Y cells. *Biochem Biophys Res Commun*. **345**, 324-331.

Jessberger, S., Nakashima, K., Clemenson, G. D., Jr., Mejia, E., Mathews, E., Ure, K., Ogawa, S., Sinton, C. M., Gage, F. H. and Hsieh, J., 2007. Epigenetic modulation of seizure-induced neurogenesis and cognitive decline. *J Neurosci*. **27**, 5967-5975.

Jia, Y. H., Zhu, X., Li, S. Y., Ni, J. H. and Jia, H. T., 2006. Kainate exposure suppresses activation of GluR2 subunit promoter in primary cultured cerebral cortical neurons through induction of RE1-silencing transcription factor. *Neurosci Lett*. **403**, 103-108.

Jiang, F. L., Tang, Y. C., Chia, S. C., Jay, T. M. and Tang, F. R., 2007. Anticonvulsive effect of a selective mGluR8 agonist (S)-3,4-dicarboxyphenylglycine (S-3,4-DCPG) in the mouse pilocarpine model of status epilepticus. *Epilepsia*. **48**, 783-792.

Jocham, G., Lezoch, K., Muller, C. P., Kart-Teke, E., Huston, J. P. and de Souza Silva, M. A., 2006. Neurokinin receptor antagonism attenuates cocaine's behavioural activating effects yet potentiates its dopamine-enhancing action in the nucleus accumbens core. *Eur J Neurosci*. **24**, 1721-1732.

Johnson, D. S., Mortazavi, A., Myers, R. M. and Wold, B., 2007. Genome-wide mapping of in vivo protein-DNA interactions. *Science*. **316**, 1497-1502.

Johnson, R., Teh, C. H., Kunarso, G., Wong, K. Y., Srinivasan, G., Cooper, M. L., Volta, M., Chan, S. S., Lipovich, L., Pollard, S. M., Karuturi, R. K., Wei, C. L., Buckley, N. J. and Stanton, L. W., 2008. REST regulates distinct transcriptional networks in embryonic and neural stem cells. *PLoS Biol*. **6**, e256.

Joshi, I. and Taylor, C. P., 2006. Pregabalin action at a model synapse: binding to presynaptic calcium channel alpha2-delta subunit reduces neurotransmission in mice. *Eur J Pharmacol*. **553**, 82-88.

Jothi, R., Cuddapah, S., Barski, A., Cui, K. and Zhao, K., 2008. Genome-wide identification of in vivo protein-DNA binding sites from ChIP-Seq data. *Nucleic Acids Res*. **36**, 5221-5231.

Jung, H. S., Kim, K. S., Chung, Y. J., Chung, H. K., Min, Y. K., Lee, M. S., Lee, M. K., Kim, K. W. and Chung, J. H., 2007. USF inhibits cell proliferation through delay in G2/M phase in FRTL-5 cells. *Endocr J*. **54**, 275-285.

Kage, R., McGregor, G. P., Thim, L. and Conlon, J. M., 1988. Neuropeptide-gamma: a peptide isolated from rabbit intestine that is derived from gamma-preprotachykinin. *J Neurochem*. **50**, 1412-1417.

Kalachikov, S., Evgrafov, O., Ross, B., Winawer, M., Barker-Cummings, C., Martinelli Boneschi, F., Choi, C., Morozov, P., Das, K., Teplitskaya, E., Yu, A., Cayanis, E., Penchaszadeh, G., Kottmann, A. H., Pedley, T. A., Hauser, W. A., Ottman, R. and Gilliam, T. C., 2002. Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nat Genet*. **30**, 335-341.

Kallunki, P., Edelman, G. M. and Jones, F. S., 1997. Tissue-specific expression of the L1 cell adhesion molecule is modulated by the neural restrictive silencer element. *J Cell Biol*. **138**, 1343-1354.

Kalynchuk, L. E., Meaney, M. J. and Kar, S., 2002. Amygdala kindling decreases insulin-like growth factor-I receptor binding sites in the rat hippocampus. *Brain Res*. **935**, 118-123.

Kaminski, R. M., Matagne, A., Leclercq, K., Gillard, M., Michel, P., Kenda, B., Talaga, P. and Klitgaard, H., 2008. SV2A protein is a broad-spectrum anticonvulsant target: functional correlation between protein binding and seizure protection in models of both partial and generalized epilepsy. *Neuropharmacology*. **54**, 715-720.

Kanazawa, I., Ogawa, T., Kimura, S. and Munekata, E., 1984. Regional distribution of substance P, neurokinin alpha and neurokinin beta in rat central nervous system. *Neurosci Res*. **2**, 111-120.

Kang, S. J., Cho, S. H., Park, K., Yi, J., Yoo, S. J. and Shin, K. S., 2008. Expression of Kir2.1 channels in astrocytes under pathophysiological conditions. *Mol Cells*. **25**, 124-130.

Kangawa, K., Minamino, N., Fukuda, A. and Matsuo, H., 1983. Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. *Biochem Biophys Res Commun*. **114**, 533-540.
Kaplan, C. D., Morris, J. R., Wu, C. and Winston, F., 2000. Spt5 and spt6 are associated with active transcription and have characteristics of general elongation factors in D. melanogaster. *Genes Dev*. **14**, 2623-2634.

Kar, S., Seto, D., Dore, S., Chabot, J. G. and Quirion, R., 1997. Systemic administration of kainic acid induces selective time dependent decrease in [125I]insulin-like growth factor I, [125I]insulin-like growth factor II and [125I]insulin receptor binding sites in adult rat hippocampal formation. *Neuroscience*. **80**, 1041-1055.

Katoh-Semba, R., Kaneko, R., Kitajima, S., Tsuzuki, M., Ichisaka, S., Hata, Y., Yamada, H., Miyazaki, N., Takahashi, Y. and Kato, K., 2009. Activation of p38 mitogen-activated protein kinase is required for in vivo brain-derived neurotrophic factor production in the rat hippocampus. *Neuroscience*.

Kelleher, R. J., 3rd, Flanagan, P. M. and Kornberg, R. D., 1990. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell*. **61**, 1209-1215.

Kiessling, M. and Gass, P., 1993. Immediate early gene expression in experimental epilepsy. *Brain Pathol*. **3**, 381-393.

Kim, S. J., Lee, B. H., Lee, Y. S. and Kang, K. S., 2007. Defective cholesterol traffic and neuronal differentiation in neural stem cells of Niemann-Pick type C disease improved by valproic acid, a histone deacetylase inhibitor. *Biochem Biophys Res Commun*. **360**, 593-599.

Kim, Y. J., Bjorklund, S., Li, Y., Sayre, M. H. and Kornberg, R. D., 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell*. **77**, 599-608.

Kimura, S., Goto, K., Ogawa, T., Sugita, Y. and Kanazawa, I., 1984. Pharmacological characterization of novel mammalian tachykinins, neurokinin alpha and neurokinin beta. *Neurosci Res*. **2**, 97-104.

Kireeva, M. L., Walter, W., Tchernajenko, V., Bondarenko, V., Kashlev, M. and Studitsky, V. M., 2002. Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. *Mol Cell*. **9**, 541-552.

Kirschbaum, B. J., Pognonec, P. and Roeder, R. G., 1992. Definition of the transcriptional activation domain of recombinant 43-kilodalton USF. *Mol Cell Biol*. **12**, 5094-5101.

Klassert, T. E., Pinto, F., Hernandez, M., Candenas, M. L., Hernandez, M. C., Abreu, J. and Almeida, T. A., 2008. Differential expression of neurokinin B and hemokinin-1 in human immune cells. *J Neuroimmunol*. **196**, 27-34.

Knezetic, J. A. and Luse, D. S., 1986. The presence of nucleosomes on a DNA template prevents initiation by RNA polymerase II in vitro. *Cell*. **45**, 95-104.

Kobayashi, T., Hirai, H., Iino, M., Fuse, I., Mitsumura, K., Washiyama, K., Kasai, S. and Ikeda, K., 2009. Inhibitory effects of the antiepileptic drug ethosuximide on G protein-activated inwardly rectifying K+ channels. *Neuropharmacology*. **56**, 499-506.

Koenigsberger, C., Chicca, J. J., 2nd, Amoureux, M. C., Edelman, G. M. and Jones, F. S., 2000. Differential regulation by multiple promoters of the gene encoding the neuron-restrictive silencer factor. *Proc Natl Acad Sci U S A*. **97**, 2291-2296.

Kojima, T., Murai, K., Naruse, Y., Takahashi, N. and Mori, N., 2001. Cell-type non-selective transcription of mouse and human genes encoding neural-restrictive silencer factor. *Brain Res Mol Brain Res*. **90**, 174-186.

Kokaia, M., Ernfors, P., Kokaia, Z., Elmer, E., Jaenisch, R. and Lindvall, O., 1995. Suppressed epileptogenesis in BDNF mutant mice. *Exp Neurol*. **133**, 215-224.

Kouzarides, T., 2007. Chromatin modifications and their function. *Cell*. **128**, 693-705.

Koyama, R. and Ikegaya, Y., 2005. To BDNF or not to BDNF: that is the epileptic hippocampus. *Neuroscientist*. **11**, 282-287.

Kramer, M. S., Cutler, N., Feighner, J., Shrivastava, R., Carman, J., Sramek, J. J., Reines, S. A., Liu, G., Snavely, D., Wyatt-Knowles, E., Hale, J. J., Mills, S. G., MacCoss, M., Swain, C. J., Harrison, T., Hill, R. G., Hefti, F., Scolnick, E. M., Cascieri, M. A., Chicchi, G. G., Sadowski, S., Williams, A. R., Hewson, L., Smith, D., Carlson, E. J., Hargreaves, R. J. and Rupniak, N. M., 1998. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. *Science*. **281**, 1640-1645.

Kumar, A., Choi, K. H., Renthal, W., Tsankova, N. M., Theobald, D. E., Truong, H. T., Russo, S. J.,

Laplant, Q., Sasaki, T. S., Whistler, K. N., Neve, R. L., Self, D. W. and Nestler, E. J., 2005. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron*. **48**, 303-314.

Kumar, S. S. and Buckmaster, P. S., 2006. Hyperexcitability, interneurons, and loss of GABAergic synapses in entorhinal cortex in a model of temporal lobe epilepsy. *J Neurosci*. **26**, 4613-4623.

Kuo, C. C., 1998. A common anticonvulsant binding site for phenytoin, carbamazepine, and lamotrigine in neuronal Na+ channels. *Mol Pharmacol*. **54**, 712-721.

Kuwahara, K., Saito, Y., Takano, M., Arai, Y., Yasuno, S., Nakagawa, Y., Takahashi, N., Adachi, Y., Takemura, G., Horie, M., Miyamoto, Y., Morisaki, T., Kuratomi, S., Noma, A., Fujiwara, H., Yoshimasa, Y., Kinoshita, H., Kawakami, R., Kishimoto, I., Nakanishi, M., Usami, S., Saito, Y., Harada, M. and Nakao, K., 2003. NRSF regulates the fetal cardiac gene program and maintains normal cardiac structure and function. *Embo J*. **22**, 6310-6321.

LaRoche, S. M. and Helmers, S. L., 2004. The new antiepileptic drugs: scientific review. *Jama*. **291**, 605-614.

Lason, W., 2001. Neurochemical and pharmacological aspects of cocaine-induced seizures. *Pol J Pharmacol*. **53**, 57-60.

Lawinger, P., Venugopal, R., Guo, Z. S., Immaneni, A., Sengupta, D., Lu, W., Rastelli, L., Marin Dias Carneiro, A., Levin, V., Fuller, G. N., Echelard, Y. and Majumder, S., 2000. The neuronal repressor REST/NRSF is an essential regulator in medulloblastoma cells. *Nat Med*. **6**, 826-831.

Lee, C. K., Shibata, Y., Rao, B., Strahl, B. D. and Lieb, J. D., 2004. Evidence for nucleosome depletion at active regulatory regions genome-wide. *Nat Genet*. **36**, 900-905.

Lee, G., Huang, Y., Washington, J. M., Briggs, N. W. and Zuo, Z., 2005. Carbamazepine enhances the activity of glutamate transporter type 3 via phosphatidylinositol 3-kinase. *Epilepsy Res*. **66**, 145-153.

Lee, J. H., Chai, Y. G. and Hersh, L. B., 2000. Expression patterns of mouse repressor element-1 silencing transcription factor 4 (REST4) and its possible function in neuroblastoma. *J Mol Neurosci*. **15**, 205-214.

Lee, J. H., Shimojo, M., Chai, Y. G. and Hersh, L. B., 2000. Studies on the interaction of REST4 with the cholinergic repressor element-1/neuron restrictive silencer element. *Brain Res Mol Brain Res*. **80**, 88-98.

Lee, M. G., Wynder, C., Cooch, N. and Shiekhattar, R., 2005. An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. *Nature*. **437**, 432-435.

Lerner, J. T., Sankar, R. and Mazarati, A. M., 2008. Galanin and epilepsy. *Cell Mol Life Sci*. **65**, 1864-1871.

Li, B., Carey, M. and Workman, J. L., 2007. The role of chromatin during transcription. *Cell*. **128**, 707-719.

Li, Y., Liu, Q., Yang, Y., Lv, Y., Chen, L., Bai, C., Nan, X., Wang, Y. and Pei, X., 2008. Regulatory role of neuron-restrictive silencing factor in the specific expression of cocaine- and amphetamine-regulated transcript gene. *J Neurochem*. **106**, 1314-1324.

Lima, A. R., Lima, M. S., Soares, B. G. and Farrell, M., 2002. Carbamazepine for cocaine dependence. *Cochrane Database Syst Rev*, CD002023.

Liu, P. S., Chiung, Y. M., Kao, Y. Y. and Chen, H. T., 2006. 2,4-Toluene diisocyanate suppressed the calcium signaling of ligand gated ion channel receptors. *Toxicology*. **219**, 167-174.

Liu, G., Yarov-Yarovoy, V., Nobbs, M., Clare, J. J., Scheuer, T. and Catterall, W. A., 2003. Differential interactions of lamotrigine and related drugs with transmembrane segment IVS6 of voltage-gated sodium channels. *Neuropharmacology*. **44**, 413-422.

Liu, H., Cao, Y., Basbaum, A. I., Mazarati, A. M., Sankar, R. and Wasterlain, C. G., 1999. Resistance to excitotoxin-induced seizures and neuronal death in mice lacking the preprotachykinin A gene. *Proc Natl Acad Sci U S A*. **96**, 12096-12101.

Liu, H., Mazarati, A. M., Katsumori, H., Sankar, R. and Wasterlain, C. G., 1999. Substance P is expressed in hippocampal principal neurons during status epilepticus and plays a critical role in the maintenance of status epilepticus. *Proc Natl Acad Sci U S A*. **96**, 5286-5291.

Liu, H., Sankar, R., Shin, D. H., Mazarati, A. M. and Wasterlain, C. G., 2000. Patterns of status epilepticus-induced substance P expression during development. *Neuroscience*. **101**, 297-304.

Livak, K. J. and Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. **25**, 402-408.

Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K. Y., Sung, K. W., Lee, C. W., Zhao, X. D., Chiu, K. P., Lipovich, L., Kuznetsov, V. A., Robson, P., Stanton, L. W., Wei, C. L., Ruan, Y., Lim, B. and Ng, H. H., 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet*. **38**, 431-440.

Lohoff, F. W., Ferraro, T. N., Sander, T., Zhao, H., Dahl, J. P., Berrettini, W. H. and Buono, R. J., 2005. No association between common variations in the human alpha 2 subunit gene (ATP1A2) of the sodium-potassium-transporting ATPase and idiopathic generalized epilepsy. *Neurosci Lett*. **382**, 33-38.

Lowenstein, D. H., Massa, S. M., Rowbotham, M. C., Collins, S. D., McKinney, H. E. and Simon, R. P., 1987. Acute neurologic and psychiatric complications associated with cocaine abuse. *Am J Med*. **83**, 841-846.

Lu, J. J., Zhang, Y. H., Pan, H., Chen, Y. C., Liu, X. Y., Jiang, Y. W., Bao, X. H., Shen, Y., Wu, H. S., Xu, K. M. and Wu, X. R., 2004. Case-control study and transmission/disequilibrium tests of the genes encoding GABRA5 and GABRB3 in a Chinese population affected by childhood absence epilepsy. *Chin Med J (Engl)*. **117**, 1497-1501.

Lucas, L. R., Hurley, D. L., Krause, J. E. and Harlan, R. E., 1992. Localization of the tachykinin neurokinin B precursor peptide in rat brain by immunocytochemistry and in situ hybridization. *Neuroscience*. **51**, 317-345.

Luger, T. A. and Lotti, T., 1998. Neuropeptides: role in inflammatory skin diseases. *J Eur Acad Dermatol Venereol*. **10**, 207-211.

Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G. and Rosenfeld, M. G., 2002. Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science*. **298**, 1747-1752.

Luo, X. and Sawadogo, M., 1996. Antiproliferative properties of the USF family of helix-loop-helix transcription factors. *Proc Natl Acad Sci U S A*. **93**, 1308-1313.

Lynch, B. A., Lambeng, N., Nocka, K., Kensel-Hammes, P., Bajjalieh, S. M., Matagne, A. and Fuks, B., 2004. The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. *Proc Natl Acad Sci U S A*. **101**, 9861-9866.

MacDonald, M. R., McCourt, D. W. and Krause, J. E., 1988. Posttranslational processing of alpha-, beta-, and gamma-preprotachykinins. Cell-free translation and early posttranslational processing events. *J Biol Chem*. **263**, 15176-15183.

MacKenzie, A., Payne, C., Boyle, S., Clarke, A. R. and Quinn, J. P., 2000. The human preprotachykinin-A gene promoter has been highly conserved and can drive human-like marker gene expression in the adult mouse CNS. *Mol Cell Neurosci*. **16**, 620-630.

MacKenzie, A. and Quinn, J., 2002. A yeast artificial chromosome containing the human preprotachykinin-A gene expresses substance P in mice and drives appropriate marker-gene expression during early brain embryogenesis. *Mol Cell Neurosci*. **19**, 72-87.

Magdalou, J., Herber, R., Bidault, R. and Siest, G., 1992. In vitro N-glucuronidation of a novel antiepileptic drug, lamotrigine, by human liver microsomes. *J Pharmacol Exp Ther*. **260**, 1166-1173.

Maggi, C. A., 2000. Principles of tachykininergic co-transmission in the peripheral and enteric nervous system. *Regul Pept*. **93**, 53-64.

Maggi, C. A., Patacchini, R., Rovero, P. and Giachetti, A., 1993. Tachykinin receptors and tachykinin receptor antagonists. *J Auton Pharmacol*. **13**, 23-93.

Magin, A., Lietz, M., Cibelli, G. and Thiel, G., 2002. RE-1 silencing transcription factor-4 (REST4) is neither a transcriptional repressor nor a de-repressor. *Neurochem Int*. **40**, 195-202.

Magloczky, Z., Wittner, L., Borhegyi, Z., Halasz, P., Vajda, J., Czirjak, S. and Freund, T. F., 2000. Changes in the distribution and connectivity of interneurons in the epileptic human dentate gyrus. *Neuroscience*. **96**, 7-25.

Maj, R., Fariello, R. G., Ukmar, G., Varasi, M., Pevarello, P., McArthur, R. A. and Salvati, P., 1998. PNU-151774E protects against kainate-induced status epilepticus and hippocampal lesions in the rat. *Eur J Pharmacol*. **359**, 27-32.

Markkanen, M., Uvarov, P. and Airaksinen, M. S., 2008. Role of upstream stimulating factors in the transcriptional regulation of the neuron-specific K-Cl cotransporter KCC2. *Brain Res*. **1236**, 8-15.

Marksteiner, J., Wahler, R., Bellmann, R., Ortler, M., Krause, J. E. and Sperk, G., 1992. Limbic seizures cause pronounced changes in the expression of neurokinin B in the hippocampus of the rat. *Neuroscience*.

49, 383-395.

Mathe, A. A., 1999. Neuropeptides and electroconvulsive treatment. *J Ect*. **15**, 60-75.

Maubach, K. A., Cody, C. and Jones, R. S., 1998. Tachykinins may modify spontaneous epileptiform activity in the rat entorhinal cortex in vitro by activating GABAergic inhibition. *Neuroscience*. **83**, 1047-1062.

Maubach, K. A., Rupniak, N. M., Kramer, M. S. and Hill, R. G., 1999. Novel strategies for pharmacotherapy of depression. *Curr Opin Chem Biol*. **3**, 481-488.

Mazarati, A. M., Wasterlain, C. G., Sankar, R. and Shin, D., 1998. Self-sustaining status epilepticus after brief electrical stimulation of the perforant path. *Brain Res*. **801**, 251-253.

Mazarati, A., Liu, H. and Wasterlain, C., 1999. Opioid peptide pharmacology and immunocytochemistry in an animal model of self-sustaining status epilepticus. *Neuroscience*. **89**, 167-173.

Mazarati, A. M., Hohmann, J. G., Bacon, A., Liu, H., Sankar, R., Steiner, R. A., Wynick, D. and Wasterlain, C. G., 2000. Modulation of hippocampal excitability and seizures by galanin. *J Neurosci*. **20**, 6276-6281.

Mazarati, A. M., Liu, H., Soomets, U., Sankar, R., Shin, D., Katsumori, H., Langel, U. and Wasterlain, C. G., 1998. Galanin modulation of seizures and seizure modulation of hippocampal galanin in animal models of status epilepticus. *J Neurosci*. **18**, 10070-10077.

Mazarati, A. M. and Wasterlain, C. G., 1999. N-methyl-D-asparate receptor antagonists abolish the maintenance phase of self-sustaining status epilepticus in rat. *Neurosci Lett*. **265**, 187-190.

McCarthy, P. W. and Lawson, S. N., 1989. Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience*. **28**, 745-753.

McNamara, J. O., 1995. Analyses of the molecular basis of kindling development. *Psychiatry Clin Neurosci*. **49**, S175-178.

Meldrum, B. S. and Chapman, A. G., 1999. Basic mechanisms of gabitril (tiagabine) and future potential developments. *Epilepsia*. **40 Suppl 9**, S2-6.

Mendelson, S. C., Morrison, C. F., McAllister, J., Paterson, J. M., Dobson, S. P., Mulderry, P. K. and Quinn, J. P., 1995. Repression of preprotachykinin-A promoter activity is mediated by a proximal promoter element. *Neuroscience*. **65**, 837-847.

Mendelson, S. C. and Quinn, J. P., 1995. Characterisation of potential regulatory elements within the rat preprotachykinin-A promoter. *Neurosci Lett*. **184**, 125-128.

Metzger, E., Wissmann, M., Yin, N., Muller, J. M., Schneider, R., Peters, A. H., Gunther, T., Buettner, R. and Schule, R., 2005. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature*. **437**, 436-439.

Miller, K. A., Witkin, J. M., Ungard, J. T. and Gasior, M., 2000. Pharmacological and behavioral

characterization of cocaine-kindled seizures in mice. *Psychopharmacology (Berl)*. **148**, 74-82.

Mistrik, P., Mader, R., Michalakis, S., Weidinger, M., Pfeifer, A. and Biel, M., 2005. The murine HCN3 gene encodes a hyperpolarization-activated cation channel with slow kinetics and unique response to cyclic nucleotides. *J Biol Chem*. **280**, 27056-27061.

Miyajima, F., Quinn, J. P., Horan, M., Pickles, A., Ollier, W. E., Pendleton, N. and Payton, A., 2008. Additive effect of BDNF and REST polymorphisms is associated with improved general cognitive ability. *Genes Brain Behav*. **7**, 714-719.

Morgan, J. I. and Curran, T., 1991. Proto-oncogene transcription factors and epilepsy. *Trends Pharmacol Sci*. **12**, 343-349.

Mosher, C. M., Tai, G. and Rettie, A. E., 2009. CYP2C9 Amino Acid Residues Influencing Phenytoin Turnover and Metabolite Regio- and Stereochemistry. *J Pharmacol Exp Ther*.

Moussaoui, S. M., Le Prado, N., Bonici, B., Faucher, D. C., Cuine, F., Laduron, P. M. and Garret, C., 1992. Distribution of neurokinin B in rat spinal cord and peripheral tissues: comparison with neurokinin A and substance P and effects of neonatal capsaicin treatment. *Neuroscience*. **48**, 969-978.

Mudo, G., Jiang, X. H., Timmusk, T., Bindoni, M. and Belluardo, N., 1996. Change in neurotrophins and their receptor mRNAs in the rat forebrain after status epilepticus induced by pilocarpine. *Epilepsia*. **37**, 198-207.

Mulligan, P., Westbrook, T. F., Ottinger, M., Pavlova, N., Chang, B., Macia, E., Shi, Y. J., Barretina, J., Liu, J., Howley, P. M., Elledge, S. J. and Shi, Y., 2008. CDYL bridges REST and histone methyltransferases for gene repression and suppression of cellular transformation. *Mol Cell*. **32**, 718-726.

Murai, K., Naruse, Y., Shaul, Y., Agata, Y. and Mori, N., 2004. Direct interaction of NRSF with TBP: chromatin reorganization and core promoter repression for neuron-specific gene transcription. *Nucleic Acids Res*. **32**, 3180-3189.

Mussap, C. J., Geraghty, D. P. and Burcher, E., 1993. Tachykinin receptors: a radioligand binding perspective. *J Neurochem*. **60**, 1987-2009.

Nair, V. D., Niznik, H. B. and Mishra, R. K., 1996. Interaction of NMDA and dopamine D2L receptors in human neuroblastoma SH-SY5Y cells. *J Neurochem*. **66**, 2390-2393.

Nakagawa, Y., Kuwahara, K., Harada, M., Takahashi, N., Yasuno, S., Adachi, Y., Kawakami, R., Nakanishi, M., Tanimoto, K., Usami, S., Kinoshita, H., Saito, Y. and Nakao, K., 2006. Class II HDACs mediate CaMK-dependent signaling to NRSF in ventricular myocytes. *J Mol Cell Cardiol*. **41**, 1010-1022.

Nakajima, T., Uchida, C., Anderson, S. F., Lee, C. G., Hurwitz, J., Parvin, J. D. and Montminy, M., 1997. RNA helicase A mediates association of CBP with RNA polymerase II. *Cell*. **90**, 1107-1112.

Namiki, K., Nakamura, A., Furuya, M., Mizuhashi, S., Matsuo, Y., Tokuhara, N., Sudo, T., Hama, H., Kuwaki, T., Yano, S., Kimura, S. and Kasuya, Y., 2007. Involvement of p38alpha in kainate-induced seizure and neuronal cell damage. *J Recept Signal Transduct Res*. **27**, 99-111.

Narlikar, G. J., Phelan, M. L. and Kingston, R. E., 2001. Generation and interconversion of multiple distinct nucleosomal states as a mechanism for catalyzing chromatin fluidity. *Mol Cell*. **8**, 1219-1230.

Nawa, H., Doteuchi, M., Igano, K., Inouye, K. and Nakanishi, S., 1984. Substance K: a novel mammalian tachykinin that differs from substance P in its pharmacological profile. *Life Sci*. **34**, 1153-1160.

Naylor, D. E., Liu, H. and Wasterlain, C. G., 2005. Trafficking of GABA(A) receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. *J Neurosci*. **25**, 7724-7733.

Naylor, D. E. and Wasterlain, C. G., 2005. GABA synapses and the rapid loss of inhibition to dentate gyrus granule cells after brief perforant-path stimulation. *Epilepsia*. **46 Suppl 5**, 142-147.

Niemeyer, M. I., Yusef, Y. R., Cornejo, I., Flores, C. A., Sepúlveda, F. V. and Cid, L. P., 2004. Functional evaluation of human ClC-2 chloride channel mutations associated with idiopathic generalized epilepsies. *Physiol Genomics*. **19**, 74-83.

Nishihara, S., Tsuda, L. and Ogura, T., 2003. The canonical Wnt pathway directly regulates NRSF/REST expression in chick spinal cord. *Biochem Biophys Res Commun*. **311**, 55-63.

Nordquist, R. E., Durkin, S., Jacquet, A. and Spooren, W., 2008. The tachykinin NK3 receptor agonist senktide induces locomotor activity in male Mongolian gerbils. *Eur J Pharmacol*. **600**, 87-92.

Nowak, M., Helleboid-Chapman, A., Jakel, H., Martin, G., Duran-Sandoval, D., Staels, B., Rubin, E. M., Pennacchio, L. A., Taskinen, M. R., Fruchart-Najib, J. and Fruchart, J. C., 2005. Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor. *Mol Cell Biol*. **25**, 1537-1548.

Nudelman, A. S., Dirocco, D. P., Lambert, T. J., Garelick, M. G., Le, J., Nathanson, N. M. and Storm, D. R., 2009. Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. *Hippocampus*.

Ocejo-Garcia, M., Baokbah, T. A., Ashurst, H. L., Cowlishaw, D., Soomro, I., Coulson, J. M. and Woll, P. J., 2005. Roles for USF-2 in lung cancer proliferation and bronchial carcinogenesis. *J Pathol*. **206**, 151-159.

Ooi, L., Belyaev, N. D., Miyake, K., Wood, I. C. and Buckley, N. J., 2006. BRG1 chromatin remodeling activity is required for efficient chromatin binding by repressor element 1-silencing transcription factor (REST) and facilitates REST-mediated repression. *J Biol Chem*. **281**, 38974-38980.

Ooi, L. and Wood, I. C., 2007. Chromatin crosstalk in development and disease: lessons from REST. *Nat Rev Genet*. **8**, 544-554.

Otsuka, M. and Yoshioka, K., 1993. Neurotransmitter functions of mammalian tachykinins. *Physiol Rev*. **73**, 229-308.

Otto, S. J., McCorkle, S. R., Hover, J., Conaco, C., Han, J. J., Impey, S., Yochum, G. S., Dunn, J. J., Goodman, R. H. and Mandel, G., 2007. A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. *J Neurosci*. **27**, 6729-6739.

Oudet, P., Gross-Bellard, M. and Chambon, P., 1975. Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. *Cell*. **4**, 281-300.

Page, N. M., Dakour, J. and Morrish, D. W., 2006. Gene regulation of neurokinin B and its receptor NK3 in late pregnancy and pre-eclampsia. *Mol Hum Reprod*. **12**, 427-433.

Page, N. M., Woods, R. J., Gardiner, S. M., Lomthaisong, K., Gladwell, R. T., Butlin, D. J., Manyonda, I. T. and Lowry, P. J., 2000. Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. *Nature*. **405**, 797-800.

Page, N. M., Woods, R. J. and Lowry, P. J., 2001. A regulatory role for neurokinin B in placental physiology and pre-eclampsia. *Regul Pept*. **98**, 97-104.

Pal, S., Nemeth, M. J., Bodine, D., Miller, J. L., Svaren, J., Thein, S. L., Lowry, P. J. and Bresnick, E. H., 2004. Neurokinin-B transcription in erythroid cells: direct activation by the hematopoietic transcription factor GATA-1. *J Biol Chem*. **279**, 31348-31356.

Palm, K., Belluardo, N., Metsis, M. and Timmusk, T., 1998. Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. *J Neurosci*. **18**, 1280-1296.

Palm, K., Metsis, M. and Timmusk, T., 1999. Neuron-specific splicing of zinc finger transcription factor REST/NRSF/XBR is frequent in neuroblastomas and conserved in human, mouse and rat. *Brain Res Mol Brain Res*. **72**, 30-39.

Parent, J. M., 2007. Adult neurogenesis in the intact and epileptic dentate gyrus. *Prog Brain Res*. **163**, 529-540.

Parent, J. M. and Murphy, G. G., 2008. Mechanisms and functional significance of aberrant seizure-induced hippocampal neurogenesis. *Epilepsia*. **49 Suppl 5**, 19-25.

Parent, J. M., Yu, T. W., Leibowitz, R. T., Geschwind, D. H., Sloviter, R. S. and Lowenstein, D. H., 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci*. **17**, 3727-3738.

Park, K. Y. and Russo, A. F., 2008. Control of the calcitonin gene-related peptide enhancer by upstream stimulatory factor in trigeminal ganglion neurons. *J Biol Chem*. **283**, 5441-5451.

Pascual, D. W., Bost, K. L., Xu-Amano, J., Kiyono, H. and McGhee, J. R., 1992. The cytokine-like action of substance P upon B cell differentiation. *Reg Immunol*. **4**, 100-104.

Patacchini, R., De Giorgio, R., Bartho, L., Barbara, G., Corinaldesi, R. and Maggi, C. A., 1998. Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *Br J Pharmacol*. **124**, 1703-1711.

Patak, E., Candenas, M. L., Pennefather, J. N., Ziccone, S., Lilley, A., Martin, J. D., Flores, C., Mantecon, A. G., Story, M. E. and Pinto, F. M., 2003. Tachykinins and tachykinin receptors in human uterus. *Br J Pharmacol*. **139**, 523-532.

Patel, H. J., Ramkissoon, S. H., Patel, P. S. and Rameshwar, P., 2005. Transformation of breast cells by

truncated neurokinin-1 receptor is secondary to activation by preprotachykinin-A peptides. *Proc Natl Acad Sci U S A*. **102**, 17436-17441.

Patel, P. D., Bochar, D. A., Turner, D. L., Meng, F., Mueller, H. M. and Pontrello, C. G., 2007. Regulation of tryptophan hydroxylase-2 gene expression by a bipartite RE-1 silencer of transcription/neuron restrictive silencing factor (REST/NRSF) binding motif. *J Biol Chem*. **282**, 26717-26724.

Paterson, J. M., Mendelson, S. C., McAllister, J., Morrison, C. F., Dobson, S., Grace, C. and Quinn, J. P., 1995. Three immediate early gene response elements in the proximal preprotachykinin-A promoter in two functionally distinct domains. *Neuroscience*. **66**, 921-932.

Paterson, J. M., Morrison, C. F., Dobson, S. P., McAllister, J. and Quinn, J. P., 1995. Characterisation of a functional E box motif in the proximal rat preprotachykinin-A promoter. *Neurosci Lett*. **191**, 185-188.

Paterson, J. M., Morrison, C. F., Mendelson, S. C., McAllister, J. and Quinn, J. P., 1995. An upstream stimulatory factor (USF) binding motif is critical for rat preprotachykinin-A promoter activity in PC12 cells. *Biochem J*. **310 (Pt 2)**, 401-406.

Pearce, R. E., Lu, W., Wang, Y., Uetrecht, J. P., Correia, M. A. and Leeder, J. S., 2008. Pathways of carbamazepine bioactivation in vitro. III. The role of human cytochrome P450 enzymes in the formation of 2,3-dihydroxycarbamazepine. *Drug Metab Dispos*. **36**, 1637-1649.

Pennefather, J. N., Lecci, A., Candenas, M. L., Patak, E., Pinto, F. M. and Maggi, C. A., 2004. Tachykinins and tachykinin receptors: a growing family. *Life Sci*. **74**, 1445-1463.

Perucca, E., 2006. Clinically relevant drug interactions with antiepileptic drugs. *Br J Clin Pharmacol*. **61**, 246-255.

Pinto, F. M., Almeida, T. A., Hernandez, M., Devillier, P., Advenier, C. and Candenas, M. L., 2004. mRNA expression of tachykinins and tachykinin receptors in different human tissues. *Eur J Pharmacol*. **494**, 233-239.

Pitkanen, A., 2002. Efficacy of current antiepileptics to prevent neurodegeneration in epilepsy models. *Epilepsy Res*. **50**, 141-160.

Pitkanen, A., Kharatishvili, I., Karhunen, H., Lukasiuk, K., Immonen, R., Nairismagi, J., Grohn, O. and Nissinen, J., 2007. Epileptogenesis in experimental models. *Epilepsia*. **48 Suppl 2**, 13-20.

Pitkanen, A. and Lukasiuk, K., 2009. Molecular and cellular basis of epileptogenesis in symptomatic epilepsy. *Epilepsy Behav*. **14 Suppl 1**, 16-25.

Pokholok, D. K., Harbison, C. T., Levine, S., Cole, M., Hannett, N. M., Lee, T. I., Bell, G. W., Walker, K., Rolfe, P. A., Herbolsheimer, E., Zeitlinger, J., Lewitter, F., Gifford, D. K. and Young, R. A., 2005. Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell*. **122**, 517-527.

Posner, E. B., Mohamed, K. and Marson, A. G., 2005. Ethosuximide, sodium valproate or lamotrigine for absence seizures in children and adolescents. *Cochrane Database Syst Rev*, CD003032.

Poulsen, F. R., Jahnsen, H., Blaabjerg, M. and Zimmer, J., 2002. Pilocarpine-induced seizure-like activity with increased BNDF and neuropeptide Y expression in organotypic hippocampal slice cultures. *Brain Res*. **950**, 103-118.

Powell, K. L., Ng, C., O'Brien, T. J., Xu, S. H., Williams, D. A., Foote, S. J. and Reid, C. A., 2008. Decreases in HCN mRNA expression in the hippocampus after kindling and status epilepticus in adult rats. *Epilepsia*. **49**, 1686-1695.

Proper, E. A., Oestreicher, A. B., Jansen, G. H., Veelen, C. W., van Rijen, P. C., Gispen, W. H. and de Graan, P. N., 2000. Immunohistochemical characterization of mossy fibre sprouting in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. *Brain*. **123 (Pt 1)**, 19-30.

Pugh, B. F. and Tjian, R., 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev*. **5**, 1935-1945.

Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R. and Kuhl, D., 1993. Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature*. **361**, 453-457.

Quinn, J. P., Bubb, V. J., Marshall-Jones, Z. V. and Coulson, J. M., 2002. Neuron restrictive silencer factor as a modulator of neuropeptide gene expression. *Regul Pept*. **108**, 135-141.

Quinn, J. P., Fiskerstrand, C. E., Gerrard, L., MacKenzie, A. and Payne, C. M., 2000. Molecular models to analyse preprotachykinin-A expression and function. *Neuropeptides*. **34**, 292-302.

Quinn, J. P., McAllister, J. and Mendelson, S., 1995. Multiple protein complexes, including AP2 and Sp1, interact with a specific site within the rat preprotachykinin-A promoter. *Biochim Biophys Acta*. **1263**, 25-34.

Quinn, J. P., Mendelson, S. C., Paterson, J. M., McAllister, J. and Morrison, C. F., 1995. Transcriptional control of neuropeptide gene expression in sensory neurons, using the preprotachykinin-A gene as a model. *Can J Physiol Pharmacol*. **73**, 957-962.

Qyang, Y., Luo, X., Lu, T., Ismail, P. M., Krylov, D., Vinson, C. and Sawadogo, M., 1999. Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation. *Mol Cell Biol*. **19**, 1508-1517.

Rada-Iglesias, A., Ameur, A., Kapranov, P., Enroth, S., Komorowski, J., Gingeras, T. R. and Wadelius, C., 2008. Whole-genome maps of USF1 and USF2 binding and histone H3 acetylation reveal new aspects of promoter structure and candidate genes for common human disorders. *Genome Res*. **18**, 380-392.
Ragsdale, D. S., McPhee, J. C., Scheuer, T. and Catterall, W. A., 1996. Common molecular determinants of local anesthetic, antiarrhythmic, and anticonvulsant block of voltage-gated Na+ channels. *Proc Natl Acad Sci U S A*. **93**, 9270-9275.

Rao, G., Patel, P. S., Idler, S. P., Maloof, P., Gascon, P., Potian, J. A. and Rameshwar, P., 2004. Facilitating role of preprotachykinin-I gene in the integration of breast cancer cells within the stromal compartment of the bone marrow: a model of early cancer progression. *Cancer Res*. **64**, 2874-2881.

Reddy, B. Y., Greco, S. J., Patel, P. S., Trzaska, K. A. and Rameshwar, P., 2009. RE-1-silencing

transcription factor shows tumor-suppressor functions and negatively regulates the oncogenic TAC1 in breast cancer cells. *Proc Natl Acad Sci U S A*. **106**, 4408-4413.

Regoli, D., Boudon, A. and Fauchere, J. L., 1994. Receptors and antagonists for substance P and related peptides. *Pharmacol Rev*. **46**, 551-599.

Reisman, D. and Rotter, V., 1993. The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the p53 tumor suppressor gene. *Nucleic Acids Res*. **21**, 345-350.

Remy, S., Urban, B. W., Elger, C. E. and Beck, H., 2003. Anticonvulsant pharmacology of voltage-gated Na+ channels in hippocampal neurons of control and chronically epileptic rats. *Eur J Neurosci*. **17**, 2648-2658.

Renthal, W., Maze, I., Krishnan, V., Covington, H. E., 3rd, Xiao, G., Kumar, A., Russo, S. J., Graham, A., Tsankova, N., Kippin, T. E., Kerstetter, K. A., Neve, R. L., Haggarty, S. J., McKinsey, T. A., Bassel-Duby, R., Olson, E. N. and Nestler, E. J., 2007. Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron*. **56**, 517-529.

Richfield, E. K., Vonsattel, J. P., MacDonald, M. E., Sun, Z., Deng, Y. P. and Reiner, A., 2002. Selective loss of striatal preprotachykinin neurons in a phenocopy of Huntington's disease. *Mov Disord*. **17**, 327-332.

Roberts, D. S., Raol, Y. H., Bandyopadhyay, S., Lund, I. V., Budreck, E. C., Passini, M. A., Wolfe, J. H., Brooks-Kayal, A. R. and Russek, S. J., 2005. Egr3 stimulation of GABRA4 promoter activity as a mechanism for seizure-induced up-regulation of GABA(A) receptor alpha4 subunit expression. *Proc Natl Acad Sci U S A*. **102**, 11894-11899.

Roder, C., Bellmann, R., McCarson, K. E., Krause, J. E. and Sperk, G., 1994. Kainic acid induced seizures cause a marked increase in the expression of neurokinin-3 receptor mRNA in the rat cerebellum. *Neurosci Lett*. **181**, 158-160.

Rodriguez, C. I., Girones, N. and Fresno, M., 2003. Cha, a basic helix-loop-helix transcription factor involved in the regulation of upstream stimulatory factor activity. *J Biol Chem*. **278**, 43135-43145.

Rogawski, M. A. and Loscher, W., 2004. The neurobiology of antiepileptic drugs. *Nat Rev Neurosci*. **5**, 553-564.

Rogawski, M. A. and Loscher, W., 2004. The neurobiology of antiepileptic drugs for the treatment of nonepileptic conditions. *Nat Med*. **10**, 685-692.

Roopra, A., Qazi, R., Schoenike, B., Daley, T. J. and Morrison, J. F., 2004. Localized domains of G9a-mediated histone methylation are required for silencing of neuronal genes. *Mol Cell*. **14**, 727-738.

Roopra, A., Sharling, L., Wood, I. C., Briggs, T., Bachfischer, U., Paquette, A. J. and Buckley, N. J., 2000. Transcriptional repression by neuron-restrictive silencer factor is mediated via the Sin3-histone deacetylase complex. *Mol Cell Biol*. **20**, 2147-2157.

Rudy, B., Chow, A., Lau, D., Amarillo, Y., Ozaita, A., Saganich, M., Moreno, H., Nadal, M. S., Hernandez-Pineda, R., Hernandez-Cruz, A., Erisir, A., Leonard, C. and Vega-Saenz de Miera, E., 1999.

Contributions of Kv3 channels to neuronal excitability. *Ann N Y Acad Sci*. **868**, 304-343.
Sanchez, R. M., Koh, S., Rio, C., Wang, C., Lamperti, E. D., Sharma, D., Corfas, G. and Jensen, F. E., 2001. Decreased glutamate receptor 2 expression and enhanced epileptogenesis in immature rat hippocampus after perinatal hypoxia-induced seizures. *J Neurosci*. **21**, 8154-8163.

Sankar, R., Shin, D. H. and Wasterlain, C. G., 1997. GABA metabolism during status epilepticus in the developing rat brain. *Brain Res Dev Brain Res*. **98**, 60-64.

Sawadogo, M. and Roeder, R. G., 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell*. **43**, 165-175.

Sawadogo, M., Van Dyke, M. W., Gregor, P. D. and Roeder, R. G., 1988. Multiple forms of the human gene-specific transcription factor USF. I. Complete purification and identification of USF from HeLa cell nuclei. *J Biol Chem*. **263**, 11985-11993.

Sawaya, M. C., Horton, R. W. and Meldrum, B. S., 1975. Effects of anticonvulsant drugs on the cerebral enzymes metabolizing GABA. *Epilepsia*. **16**, 649-655.

Scharfman, H. E., Goodman, J. H. and Sollas, A. L., 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci*. **20**, 6144-6158.

Schiltz, R. L., Mizzen, C. A., Vassilev, A., Cook, R. G., Allis, C. D. and Nakatani, Y., 1999. Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. *J Biol Chem*. **274**, 1189-1192.

Schoenherr, C. J. and Anderson, D. J., 1995. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. *Science*. **267**, 1360-1363.

Schroder, R. L., Jespersen, T., Christophersen, P., Strobaek, D., Jensen, B. S. and Olesen, S. P., 2001. KCNQ4 channel activation by BMS-204352 and retigabine. *Neuropharmacology*. **40**, 888-898.

Schwarzer, C., Williamson, J. M., Lothman, E. W., Vezzani, A. and Sperk, G., 1995. Somatostatin, neuropeptide Y, neurokinin B and cholecystokinin immunoreactivity in two chronic models of temporal lobe epilepsy. *Neuroscience*. **69**, 831-845.

Seo, T., Nakada, N., Ueda, N., Hagiwara, T., Hashimoto, N., Nakagawa, K. and Ishitsu, T., 2006. Effect of CYP3A5*3 on carbamazepine pharmacokinetics in Japanese patients with epilepsy. *Clin Pharmacol Ther*. **79**, 509-510.

Sepkuty, J. P., Cohen, A. S., Eccles, C., Rafiq, A., Behar, K., Ganel, R., Coulter, D. A. and Rothstein, J. D., 2002. A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. *J Neurosci*. **22**, 6372-6379.

Seress, L., Abraham, H., Doczi, T., Lazar, G. and Kozicz, T., 2004. Cocaine- and amphetamine-regulated transcript peptide (CART) is a selective marker of rat granule cells and of human mossy cells in the hippocampal dentate gyrus. *Neuroscience*. **125**, 13-24.

Severini, C., Improta, G., Falconieri-Erspamer, G., Salvadori, S. and Erspamer, V., 2002. The tachykinin

peptide family. *Pharmacol Rev*. **54**, 285-322.
Sha, M., Ferre-D'Amare, A. R., Burley, S. K. and Goss, D. J., 1995. Anti-cooperative biphasic equilibrium binding of transcription factor upstream stimulatory factor to its cognate DNA monitored by protein fluorescence changes. *J Biol Chem*. **270**, 19325-19329.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A. and Shi, Y., 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell*. **119**, 941-953.

Shimojo, M., 2006. Characterization of the nuclear targeting signal of REST/NRSF. *Neurosci Lett*. **398**, 161-166.

Shimojo, M., 2008. Huntingtin regulates RE1-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) nuclear trafficking indirectly through a complex with REST/NRSF-interacting LIM domain protein (RILP) and dynactin p150 Glued. *J Biol Chem*. **283**, 34880-34886.

Shimojo, M. and Hersh, L. B., 2003. REST/NRSF-interacting LIM domain protein, a putative nuclear translocation receptor. *Mol Cell Biol*. **23**, 9025-9031.

Shimojo, M. and Hersh, L. B., 2004. Regulation of the cholinergic gene locus by the repressor element-1 silencing transcription factor/neuron restrictive silencer factor (REST/NRSF). *Life Sci*. **74**, 2213-2225.

Shimojo, M. and Hersh, L. B., 2006. Characterization of the REST/NRSF-interacting LIM domain protein (RILP): localization and interaction with REST/NRSF. *J Neurochem*. **96**, 1130-1138.

Shimojo, M., Lee, J. H. and Hersh, L. B., 2001. Role of zinc finger domains of the transcription factor neuron-restrictive silencer factor/repressor element-1 silencing transcription factor in DNA binding and nuclear localization. *J Biol Chem*. **276**, 13121-13126.

Shimojo, M., Paquette, A. J., Anderson, D. J. and Hersh, L. B., 1999. Protein kinase A regulates cholinergic gene expression in PC12 cells: REST4 silences the silencing activity of neuron-restrictive silencer factor/REST. *Mol Cell Biol*. **19**, 6788-6795.

Singh, S. K., Kagalwala, M. N., Parker-Thornburg, J., Adams, H. and Majumder, S., 2008. REST maintains self-renewal and pluripotency of embryonic stem cells. *Nature*. **453**, 223-227.

Sirito, M., Lin, Q., Deng, J. M., Behringer, R. R. and Sawadogo, M., 1998. Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice. *Proc Natl Acad Sci U S A*. **95**, 3758-3763.

Sirito, M., Lin, Q., Maity, T. and Sawadogo, M., 1994. Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells. *Nucleic Acids Res*. **22**, 427-433.

Sirito, M., Walker, S., Lin, Q., Kozlowski, M. T., Klein, W. H. and Sawadogo, M., 1992. Members of the USF family of helix-loop-helix proteins bind DNA as homo- as well as heterodimers. *Gene Expr*. **2**, 231-240.

Sitges, M., Chiu, L. M., Guarneros, A. and Nekrassov, V., 2007. Effects of carbamazepine, phenytoin, lamotrigine, oxcarbazepine, topiramate and vinpocetine on Na+ channel-mediated release of [3H]glutamate in hippocampal nerve endings. *Neuropharmacology*. **52**, 598-605.

Spencer, E. M., Chandler, K. E., Haddley, K., Howard, M. R., Hughes, D., Belyaev, N. D., Coulson, J. M., Stewart, J. P., Buckley, N. J., Kipar, A., Walker, M. C. and Quinn, J. P., 2006. Regulation and role of REST and REST4 variants in modulation of gene expression in in vivo and in vitro in epilepsy models. *Neurobiol Dis*. **24**, 41-52.

Sperk, G., Marksteiner, J., Saria, A. and Humpel, C., 1990. Differential changes in tachykinins after kainic acid-induced seizures in the rat. *Neuroscience*. **34**, 219-224.

Sperk, G., Wieser, R., Widmann, R. and Singer, E. A., 1986. Kainic acid induced seizures: changes in somatostatin, substance P and neurotensin. *Neuroscience*. **17**, 1117-1126.

Splawski, I., Shen, J., Timothy, K. W., Lehmann, M. H., Priori, S., Robinson, J. L., Moss, A. J., Schwartz, P. J., Towbin, J. A., Vincent, G. M. and Keating, M. T., 2000. Spectrum of mutations in long-QT syndrome genes. KVLQT1, HERG, SCN5A, KCNE1, and KCNE2. *Circulation*. **102**, 1178-1185.

Stefani, A., Spadoni, F., Siniscalchi, A. and Bernardi, G., 1996. Lamotrigine inhibits Ca2+ currents in cortical neurons: functional implications. *Eur J Pharmacol*. **307**, 113-116.

Steiger, J. L., Bandyopadhyay, S., Farb, D. H. and Russek, S. J., 2004. cAMP response element-binding protein, activating transcription factor-4, and upstream stimulatory factor differentially control hippocampal GABABR1a and GABABR1b subunit gene expression through alternative promoters. *J Neurosci*. **24**, 6115-6126.

Steiner, T. J., Dellaportas, C. I., Findley, L. J., Gross, M., Gibberd, F. B., Perkin, G. D., Park, D. M. and Abbott, R., 1999. Lamotrigine monotherapy in newly diagnosed untreated epilepsy: a double-blind comparison with phenytoin. *Epilepsia*. **40**, 601-607.

Stenfors, C., Srinivasan, G. R., Theodorsson, E. and Mathe, A. A., 1992. Electroconvulsive stimuli and brain peptides: effect of modification of seizure duration on neuropeptide Y, neurokinin A, substance P and neurotensin. *Brain Res*. **596**, 251-258.

Sterner, D. E. and Berger, S. L., 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev*. **64**, 435-459.

Stillitano, F., Lonardo, G., Zicha, S., Varro, A., Cerbai, E., Mugelli, A. and Nattel, S., 2008. Molecular basis of funny current (If) in normal and failing human heart. *J Mol Cell Cardiol*. **45**, 289-299.

Strahl, B. D., Grant, P. A., Briggs, S. D., Sun, Z. W., Bone, J. R., Caldwell, J. A., Mollah, S., Cook, R. G., Shabanowitz, J., Hunt, D. F. and Allis, C. D., 2002. Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. *Mol Cell Biol*. **22**, 1298-1306.

Su, X., Gopalakrishnan, V., Stearns, D., Aldape, K., Lang, F. F., Fuller, G., Snyder, E., Eberhart, C. G. and Majumder, S., 2006. Abnormal expression of REST/NRSF and Myc in neural stem/progenitor cells causes cerebellar tumors by blocking neuronal differentiation. *Mol Cell Biol*. **26**, 1666-1678.

Su, X., Kameoka, S., Lentz, S. and Majumder, S., 2004. Activation of REST/NRSF target genes in neural stem cells is sufficient to cause neuronal differentiation. *Mol Cell Biol*. **24**, 8018-8025.

Sun, Y. M., Cooper, M., Finch, S., Lin, H. H., Chen, Z. F., Williams, B. P. and Buckley, N. J., 2008. Rest-

mediated regulation of extracellular matrix is crucial for neural development. *PLoS ONE*. **3**, e3656.
Sun, Y. M., Greenway, D. J., Johnson, R., Street, M., Belyaev, N. D., Deuchars, J., Bee, T., Wilde, S. and Buckley, N. J., 2005. Distinct profiles of REST interactions with its target genes at different stages of neuronal development. *Mol Biol Cell*. **16**, 5630-5638.

Sun, D. and Murali, S. G., 1998. Stimulation of Na+-K+-2Cl- cotransporter in neuronal cells by excitatory neurotransmitter glutamate. *Am J Physiol*. **275**, C772-779.

Szot, P., Weinshenker, D., White, S. S., Robbins, C. A., Rust, N. C., Schwartzkroin, P. A. and Palmiter, R. D., 1999. Norepinephrine-deficient mice have increased susceptibility to seizure-inducing stimuli. *J Neurosci*. **19**, 10985-10992.

Tabuchi, A., Sakaya, H., Kisukeda, T., Fushiki, H. and Tsuda, M., 2002. Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. *J Biol Chem*. **277**, 35920-35931.

Tabuchi, A., Yamada, T., Sasagawa, S., Naruse, Y., Mori, N. and Tsuda, M., 2002. REST4-mediated modulation of REST/NRSF-silencing function during BDNF gene promoter activation. *Biochem Biophys Res Commun*. **290**, 415-420.

Tahiliani, M., Mei, P., Fang, R., Leonor, T., Rutenberg, M., Shimizu, F., Li, J., Rao, A. and Shi, Y., 2007. The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation. *Nature*. **447**, 601-605.

Takeda, Y., Chou, K. B., Takeda, J., Sachais, B. S. and Krause, J. E., 1991. Molecular cloning, structural characterization and functional expression of the human substance P receptor. *Biochem Biophys Res Commun*. **179**, 1232-1240.

Taniura, H., Sng, J. C. and Yoneda, Y., 2006. Histone modifications in status epilepticus induced by kainate. *Histol Histopathol*. **21**, 785-791.

Tapia-Ramirez, J., Eggen, B. J., Peral-Rubio, M. J., Toledo-Aral, J. J. and Mandel, G., 1997. A single zinc finger motif in the silencing factor REST represses the neural-specific type II sodium channel promoter. *Proc Natl Acad Sci U S A*. **94**, 1177-1182.

Tasker, J. G. and Dudek, F. E., 1991. Electrophysiology of GABA-mediated synaptic transmission and possible roles in epilepsy. *Neurochem Res*. **16**, 251-262.

Tate, S. K., Depondt, C., Sisodiya, S. M., Cavalleri, G. L., Schorge, S., Soranzo, N., Thom, M., Sen, A., Shorvon, S. D., Sander, J. W., Wood, N. W. and Goldstein, D. B., 2005. Genetic predictors of the maximum doses patients receive during clinical use of the anti-epileptic drugs carbamazepine and phenytoin. *Proc Natl Acad Sci U S A*. **102**, 5507-5512.

Tatemoto, K., Lundberg, J. M., Jornvall, H. and Mutt, V., 1985. Neuropeptide K: isolation, structure and biological activities of a novel brain tachykinin. *Biochem Biophys Res Commun*. **128**, 947-953.
Tateno, T., Izumiyama, H., Doi, M., Yoshimoto, T., Shichiri, M., Inoshita, N., Oyama, K., Yamada, S. and Hirata, Y., 2007. Differential gene expression in ACTH -secreting and non-functioning pituitary tumors. *Eur J Endocrinol*. **157**, 717-724.

Thiry, A., Dogne, J. M., Supuran, C. T. and Masereel, B., 2007. Carbonic anhydrase inhibitors as anticonvulsant agents. *Curr Top Med Chem*. **7**, 855-864.

Timmusk, T., Palm, K., Lendahl, U. and Metsis, M., 1999. Brain-derived neurotrophic factor expression in vivo is under the control of neuron-restrictive silencer element. *J Biol Chem*. **274**, 1078-1084.

Tirode, F., Busso, D., Coin, F. and Egly, J. M., 1999. Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. *Mol Cell*. **3**, 87-95.
Topaloglu, A. K., Reimann, F., Guclu, M., Yalin, A. S., Kotan, L. D., Porter, K. M., Serin, A., Mungan, N. O., Cook, J. R., Ozbek, M. N., Imamoglu, S., Akalin, N. S., Yuksel, B., O'Rahilly, S. and Semple, R. K., 2009. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet*. **41**, 354-358.

Tornberg, J., Voikar, V., Savilahti, H., Rauvala, H. and Airaksinen, M. S., 2005. Behavioural phenotypes of hypomorphic KCC2-deficient mice. *Eur J Neurosci*. **21**, 1327-1337.

Tsankova, N. M., Kumar, A. and Nestler, E. J., 2004. Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. *J Neurosci*. **24**, 5603-5610.

Vakoc, C. R., Mandat, S. A., Olenchock, B. A. and Blobel, G. A., 2005. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol Cell*. **19**, 381-391.

Valouev, A., Johnson, D. S., Sundquist, A., Medina, C., Anton, E., Batzoglou, S., Myers, R. M. and Sidow, A., 2008. Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. *Nat Methods*. **5**, 829-834.

van Deursen, D., van Leeuwen, M., Vaulont, S., Jansen, H. and Verhoeven, A. J., 2009. Upstream Stimulatory Factors 1 and 2 activate the human hepatic lipase promoter via E-box dependent and independent mechanisms. *Biochim Biophys Acta*. **1791**, 229-237.

Van Gelder, R. N., von Zastrow, M. E., Yool, A., Dement, W. C., Barchas, J. D. and Eberwine, J. H., 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci U S A*. **87**, 1663-1667.

Vaquero, A., Scher, M. B., Lee, D. H., Sutton, A., Cheng, H. L., Alt, F. W., Serrano, L., Sternglanz, R. and Reinberg, D., 2006. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev*. **20**, 1256-1261.

Varghese, A., Tenbroek, E. M., Coles, J., Jr. and Sigg, D. C., 2006. Endogenous channels in HEK cells and potential roles in HCN ionic current measurements. *Prog Biophys Mol Biol*. **90**, 26-37.

Vettese-Dadey, M., Grant, P. A., Hebbes, T. R., Crane- Robinson, C., Allis, C. D. and Workman, J. L., 1996. Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA in vitro. *Embo J*. **15**, 2508-2518.

Viollet, B., Lefrancois-Martinez, A. M., Henrion, A., Kahn, A., Raymondjean, M. and Martinez, A., 1996. Immunochemical characterization and transacting properties of upstream stimulatory factor isoforms. *J Biol Chem*. **271**, 1405-1415.

Vo, N. and Goodman, R. H., 2001. CREB-binding protein and p300 in transcriptional regulation. *J Biol Chem*. **276**, 13505-13508.

von Euler, U. S. G., J. H., 1931. An unidentified derepressor substance in certain tissue extracts. *Journal of Physiology* **72**, 74-87.

Walker, M. C., Ruiz, A. and Kullmann, D. M., 2001. Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. *Neuron*. **29**, 703-715.

Wang, S. J., Huang, C. C., Hsu, K. S., Tsai, J. J. and Gean, P. W., 1996. Presynaptic inhibition of excitatory neurotransmission by lamotrigine in the rat amygdalar neurons. *Synapse*. **24**, 248-255.

Wang, W., Carey, M. and Gralla, J. D., 1992. Polymerase II promoter activation: closed complex formation and ATP-driven start site opening. *Science*. **255**, 450-453.

Wang, Z., Zang, C., Rosenfeld, J. A., Schones, D. E., Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Peng, W., Zhang, M. Q. and Zhao, K., 2008. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet*. **40**, 897-903.

Warrington, J., 2000. Large-scale genomic analysis using Affymetrix GeneChip(R) probe arrays. *Microarray Biochip Technology*, 119-148.

Warrington, J. A., Nair, A., Mahadevappa, M. and Tsyganskaya, M., 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiol Genomics*. **2**, 143-147.

Warter, J. M., Coquillat, G. and Kurtz, D., 1975. Human circulating dopamine-beta-hydroxylase and epilepsy. *Psychopharmacologia*. **41**, 75-79.

Wasterlain, C. G. and Chen, J. W., 2008. Mechanistic and pharmacologic aspects of status epilepticus and its treatment with new antiepileptic drugs. *Epilepsia*. **49 Suppl 9**, 63-73.

Wasterlain, C. G., Liu, H., Mazarati, A. M., Baldwin, R. A., Shirasaka, Y., Katsumori, H., Thompson, K. W., Sankar, R., Pereira de Vasconselos, A. and Nehlig, A., 2000. Self-sustaining status epilepticus: a condition maintained by potentiation of glutamate receptors and by plastic changes in substance P and other peptide neuromodulators. *Epilepsia*. **41 Suppl 6**, S134-143.

Wasterlain, C. G., Mazarati, A. M., Naylor, D., Niquet, J., Liu, H., Suchomelova, L., Baldwin, R., Katsumori, H., Shirasaka, Y., Shin, D. and Sankar, R., 2002. Short-term plasticity of hippocampal neuropeptides and neuronal circuitry in experimental status epilepticus. *Epilepsia*. **43 Suppl 5**, 20-29.

Wasterlain, C. G., Niquet, J., Thompson, K. W., Baldwin, R., Liu, H., Sankar, R., Mazarati, A. M., Naylor, D., Katsumori, H., Suchomelova, L. and Shirasaka, Y., 2002. Seizure-induced neuronal death in the immature brain. *Prog Brain Res*. **135**, 335-353.

Wasterlain, C. G., Shirasaka, Y., Mazarati, A. M. and Spigelman, I., 1996. Chronic epilepsy with damage restricted to the hippocampus: possible mechanisms. *Epilepsy Res*. **26**, 255-265.

Watanabe, H., Mizutani, T., Haraguchi, T., Yamamichi, N., Minoguchi, S., Yamamichi-Nishina, M., Mori, N., Kameda, T., Sugiyama, T. and Iba, H., 2006. SWI/SNF complex is essential for NRSF-mediated suppression of neuronal genes in human nonsmall cell lung carcinoma cell lines. *Oncogene*. **25**, 470-479.

Watanabe, Y., Kameoka, S., Gopalakrishnan, V., Aldape, K. D., Pan, Z. Z., Lang, F. F. and Majumder, S., 2004. Conversion of myoblasts to physiologically active neuronal phenotype. *Genes Dev*. **18**, 889-900.

Weinshenker, D. and Szot, P., 2002. The role of catecholamines in seizure susceptibility: new results using genetically engineered mice. *Pharmacol Ther*. **94**, 213-233.

West, A. G., Huang, S., Gaszner, M., Litt, M. D. and Felsenfeld, G., 2004. Recruitment of histone modifications by USF proteins at a vertebrate barrier element. *Mol Cell*. **16**, 453-463.

Westbrook, T. F., Hu, G., Ang, X. L., Mulligan, P., Pavlova, N. N., Liang, A., Leng, Y., Maehr, R., Shi, Y., Harper, J. W. and Elledge, S. J., 2008. SCFbeta-TRCP controls oncogenic transformation and neural differentiation through REST degradation. *Nature*. **452**, 370-374.

Westbrook, T. F., Martin, E. S., Schlabach, M. R., Leng, Y., Liang, A. C., Feng, B., Zhao, J. J., Roberts, T. M., Mandel, G., Hannon, G. J., Depinho, R. A., Chin, L. and Elledge, S. J., 2005. A genetic screen for candidate tumor suppressors identifies REST. *Cell*. **121**, 837-848.

Whelan, H. T., Bajic, D. M., Karlovits, S. M., Houle, J. M. and Kindwall, E. P., 1998. Use of cytochrome-P450 mono-oxygenase 2 E1 isozyme inhibitors to delay seizures caused by central nervous system oxygen toxicity. *Aviat Space Environ Med*. **69**, 480-485.

Willow, M., Gonoi, T. and Catterall, W. A., 1985. Voltage clamp analysis of the inhibitory actions of diphenylhydantoin and carbamazepine on voltage-sensitive sodium channels in neuroblastoma cells. *Mol Pharmacol*. **27**, 549-558.

Wirbelauer, C., Bell, O. and Schubeler, D., 2005. Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active histone modifications show a promoter-proximal bias. *Genes Dev*. **19**, 1761-1766.

Witkin, J., Gasior, M., Acri, J., Beekman, M., Thurkauf, A., Yuan, J., DeBoer, P., Wikstrom, H. and Dijkstra, D., 1998. Atypical antipsychotic-like effects of the dopamine D3 receptor agonist, (+)-PD 128,907. *Eur J Pharmacol*. **347**, R1-3.

Wood, I. C., Belyaev, N. D., Bruce, A. W., Jones, C., Mistry, M., Roopra, A. and Buckley, N. J., 2003. Interaction of the repressor element 1-silencing transcription factor (REST) with target genes. *J Mol Biol*. **334**, 863-874.

Wood, I. C., Roopra, A. and Buckley, N. J., 1996. Neural specific expression of the m4 muscarinic acetylcholine receptor gene is mediated by a RE1/NRSE-type silencing element. *J Biol Chem*. **271**, 14221-14225.

Workman, J. L., 2006. Nucleosome displacement in transcription. *Genes Dev*. **20**, 2009-2017.

Wu, J. and Xie, X., 2006. Comparative sequence analysis reveals an intricate network among REST,

CREB and miRNA in mediating neuronal gene expression. *Genome Biol*. **7**, R85.

Xiao, Q., Kenessey, A. and Ojamaa, K., 2002. Role of USF1 phosphorylation on cardiac alpha-myosin heavy chain promoter activity. *Am J Physiol Heart Circ Physiol*. **283**, H213-219.

Xie, X., Lancaster, B., Peakman, T. and Garthwaite, J., 1995. Interaction of the antiepileptic drug lamotrigine with recombinant rat brain type IIA Na+ channels and with native Na+ channels in rat hippocampal neurones. *Pflugers Arch*. **430**, 437-446.

Yang, J., Wetterstrand, C. and Jones, R. S., 2007. Felbamate but not phenytoin or gabapentin reduces glutamate release by blocking presynaptic NMDA receptors in the entorhinal cortex. *Epilepsy Res*. **77**, 157-164.

Yang, Y., Li, Y., Lv, Y., Zhang, S., Chen, L., Bai, C., Nan, X., Yue, W. and Pei, X., 2008. NRSF silencing induces neuronal differentiation of human mesenchymal stem cells. *Exp Cell Res*. **314**, 2257-2265.

Yeo, M., Lee, S. K., Lee, B., Ruiz, E. C., Pfaff, S. L. and Gill, G. N., 2005. Small CTD phosphatases function in silencing neuronal gene expression. *Science*. **307**, 596-600.

Yoshida, S., Okada, M., Zhu, G. and Kaneko, S., 2007. Carbamazepine prevents breakdown of neurotransmitter release induced by hyperactivation of ryanodine receptor. *Neuropharmacology*. **52**, 1538-1546.

You, A., Tong, J. K., Grozinger, C. M. and Schreiber, S. L., 2001. CoREST is an integral component of the CoREST- human histone deacetylase complex. *Proc Natl Acad Sci U S A*. **98**, 1454-1458.

Zachrisson, O., Lindefors, N. and Brene, S., 1998. A tachykinin NK1 receptor antagonist, CP-122,721-1, attenuates kainic acid-induced seizure activity. *Brain Res Mol Brain Res*. **60**, 291-295.

Zahn, R. K., Tolner, E. A., Derst, C., Gruber, C., Veh, R. W. and Heinemann, U., 2008. Reduced ictogenic potential of 4-aminopyridine in the perirhinal and entorhinal cortex of kainate-treated chronic epileptic rats. *Neurobiol Dis*. **29**, 186-200.

Zhang, D., Zhang, L., Lou, D. W., Nakabeppu, Y., Zhang, J. and Xu, M., 2002. The dopamine D1 receptor is a critical mediator for cocaine-induced gene expression. *J Neurochem*. **82**, 1453-1464.

Zhang, X., Andren, P. E., Chergui, K. and Svenningsson, P., 2008. Neurokinin B/NK3 receptors exert feedback inhibition on L-DOPA actions in the 6-OHDA lesion rat model of Parkinson's disease. *Neuropharmacology*. **54**, 1143-1152.

Zhang, Y., Lu, L., Furlonger, C., Wu, G. E. and Paige, C. J., 2000. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. *Nat Immunol*. **1**, 392-397.

Zimber, A., Nguyen, Q. D. and Gespach, C., 2004. Nuclear bodies and compartments: functional roles and cellular signalling in health and disease. *Cell Signal*. **16**, 1085-1104.

Zuccato, C., Belyaev, N., Conforti, P., Ooi, L., Tartari, M., Papadimou, E., MacDonald, M., Fossale, E., Zeitlin, S., Buckley, N. and Cattaneo, E., 2007. Widespread disruption of repressor element-1 silencing

transcription factor/neuron-restrictive silencer factor occupancy at its target genes in Huntington's disease. *J Neurosci*. **27**, 6972-6983.

Zuccato, C., Tartari, M., Crotti, A., Goffredo, D., Valenza, M., Conti, L., Cataudella, T., Leavitt, B. R., Hayden, M. R., Timmusk, T., Rigamonti, D. and Cattaneo, E., 2003. Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet*. **35**, 76-83.

Appendix Section

Appendix 1

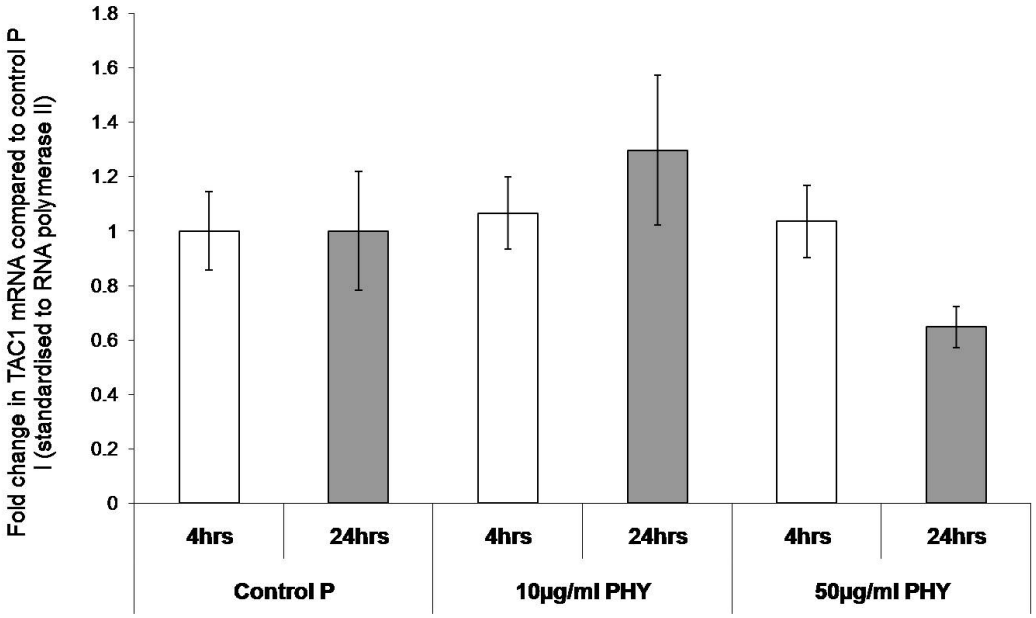

Figure A1. Phenytoin treatment has nil affect on TAC1 expression. qPCR analysis of TAC1 mRNA expression in response to PHY treatment in human SK-N-AS neuroblastoma cells. TAC1-expressing SK-N-AS cells were treated with either 10µg/ml or 50µg/ml PHY, or vehicle control (Control P), for 4hrs or 24hrs, before being processed for RNA extraction. mRNA expression of TAC1 was analysed using qPCR, standardised against the house-keeping gene Pol-II, with mean fold changes compared to vehicle controls given. Standard errors are given as y-axis error bars. Students *t*-test was performed. No significant changes compared to vehicle controls were observed.

Appendix 2

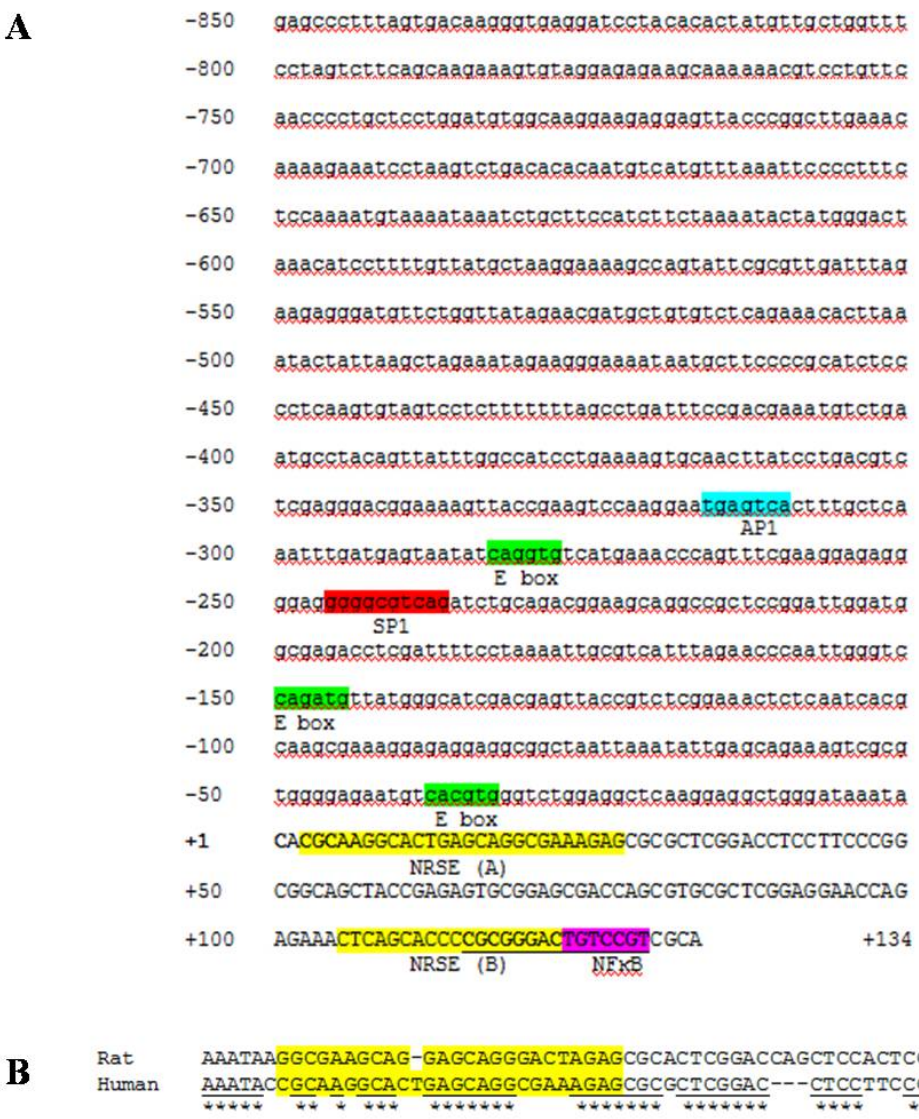

Figure A2. (A) Human TAC1 proximal promoter spanning –850 to +134. Putative TF binding sites are highlighted. E box motifs based on the canonical sequence CANNTG are highlighted in green. A putative SP1 binding site based on the consensus sequence (G/T)(G/A)GGCG(G/T)(G/A)(G/A)(C/T) is highlighted in red. Putative AP1 binding sites based on sequence TGA(G/C)TCA are highlighted in light blue. The NFκB binding motif is highlighted in pink, and underlined to distinguish overlapping sequences (Greco *et al*., 2007). Two predicted NRSEs are highlighted in yellow. NRSE (A) is a putative NRSE based upon alignment with the characterised rat TAC1 NRSE, whilst NRSE (B) is the predicted NRSE from Greco *et al*., 2007. **(B)** Alignment of Rat and human TAC1 promoter regions, focused on the characterised rat NRSE aligned against human. This alignment revealed the putative NRSE (A)

Appendix 3

Figure A.3 Sequence alignment between rat and human TAC1 promoter regions. The human TAC1 promoter region was aligned against the rat TAC1 promoter region, and the rat region spanning -325 to +98 is given. The alignment reveals that the rat TAC1 NRSE (highlighted yellow) aligns well with the human TAC1 sequence, with a resulting putative human TAC1 NRSE exhibiting 65% homology to the characterised rat TAC1 NRSE (17/26). In contrast the previously characterised human TAC1 NRSE, proposed by Greco *et al* (2007) (highlighted in light blue), is poorly conserved in the rat. The rodent E box motifs located at -60, -170 and -308 are perfectly (100%) conserved in the human TAC1 sequence. However the E boxes at +61 and +69 are poorly conserved, with only 50% homology and 33% homology between the rat and human sequences, respectively. The E box motifs are highlighted in green.

Appendix 4

Rat TAC1:	**Human TAC1:**
E box motifs: +61 to +67 (115bp) *to -54* **-60 to -54** **(104bp)** *to -164* **-170 to -164** **(132bp)** *to -302* -308 to -302	E box motifs: **-38 to -32** **(106bp)** *to -144* **-150 to -144** **(132bp)** *to -282* -288 to -282
NRSE: **-21 to +4**	NRSE (A): **+2 to +28**
Distance between NRSE and E boxes: **-170 E box and NRSE = 143bp** **-60 E box and NRSE = 33bp** +60 E box and NRSE = 57bp	Distance between NRSE (A) and E boxes: **-38 E box and NRSE = 34bp** **-150 E box and NRSE = 146bp**

Table A1. Comparison of E box motifs and putative NRSEs in rat and human TAC1 proximal promoter regions. Table gives the positions of previously characterised or putative E box motifs and NRSE within the rat and human TAC1 proximal promoter regions, with positions given corresponding to TSS. Distances between E box motifs are given in red, whilst distances between the (characterised or putative) NRSE and stated E box, is given in blue. Similarities between the rat and human TAC1 promoter regions are highlighted in bold.

Appendix 5

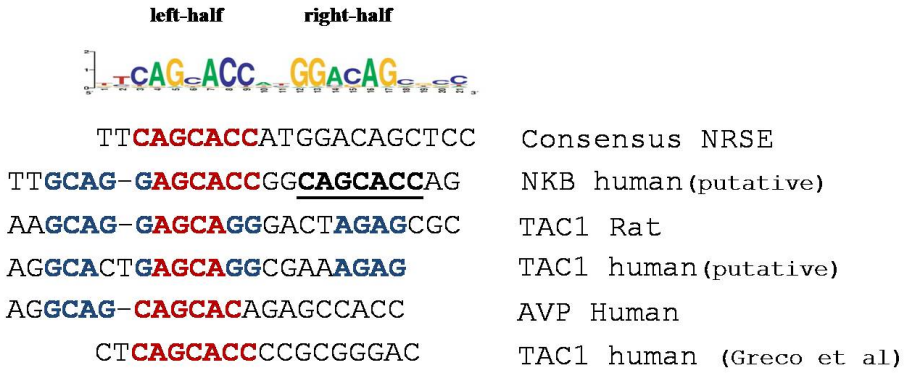

Figure A4. Comparison of neuropeptide NRSEs to consensus reveals homology to the left-half site. Sequence comparison of the NRSEs in NKB (human, putative), TAC1 (rat, characterised; human, putative; human; characterised) and AVP (human, characterised), reveals strong homology between the neuropeptide NRSEs and the left-half site of the consensus NRSE (CAGCACC). Furthermore, the alignment reveals that the NKB sequence has two matches to the left-half site, with the second highlighted in black bold, and underlined. Homology between the neuropeptide sequence and the consensus sequence is highlighted in red, whilst homology between the neuropeptide sequences is highlighted in blue.